Helicobacter suis and the stomach-brain axis: epidemiological, pathogenetic and diagnostic aspects

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List of abbreviations

AGS	human gastric adenocarcinoma cells
AhpC	alkyl hydroperoxide reductase
AlpA/B	adherence-associated lipoprotein A/B
AnsB	l-asparginase II
aPKC	atypical protein kinase C
BabA	blood group antigen binding adhesion A
BBB	blood-brain barrier
Blood-CSF barrier	blood-cerebrospinal fluid barrier
CagA	cytotoxin-associated gene A
CagPAI	cag pathogenicity island
CarR	non-coding gene in the genome of *Helicobacter suis*
Che	chemotaxis protein
CNS	central nervous system
Cldn	Claudin
COX	cyclo-oxygenase
CPE	choroid plexus epithelium
CSF	cerebrospinal fluid
CXCL	chemokine ligand
DC	dendritic cell
DNA	deoxyribonucleic acid
ECIS	electrical cell impedance system
EGF	epithelial growth factor
EIA	enzyme immune assay
ErbB	epidermal growth factor receptors
ERK	extracellular signal-regulated kinase
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FlaA/B	flagellin A/B
FldA	flavodoxin protein
FlgD	flagellar cap
FlgE	flagellar hook
FOXP3	forkhead box protein P3
Ggt	gamma-glutamyl transpeptidase
HBSS	Hank's balanced salt solution
HCL	hydrochloric acid
HE	hematoxylin and eosin
Hof	*Helicobacter* outer membrane protein family

Hom	*Helicobacter* outer membrane
Hop	*Helicobacter* outer membrane porin
Hor	hop related protein
HP-NAP	*Helicobacter pylori* neutrophil-activating protein
Hsp60	heat shock protein 60
HtrA	high temperature requirement A
IBD	inflammatory bowel disease
ICA	immunochromatography assay
IceA	ulcer-associated protein restriction endonuclease
IFN	Interferon
IL	Interleukin
iNos	inducible nitric oxide synthase
IP	idiopathic parkinsonism
KatA	Catalase
KC	keratinocyte-derived chemokine
Levadopa	l-3,4-dihydroxyphenylalanine
LIX	LPS-induced CXC chemokine
LPS	Lipopolysaccharide
LRRK2	leuncin-rich repeat kinase 2
MALT	mucosa-associated lymphoid tissue
MAP	mitogen-activating protein
MIC	minimum inhibitory concentration
MIP	macrophage inflammatory protein
MLST	multi-locus sequence typing
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridin
mRNA	messenger ribonucleic acid
MRPS6	mitochondrial ribosomal protein S6
Muc	Mucin
NADPH	nicotinamide adenine dinucleotide phosphate
NAP	neutrophil-activating protein
ND	not detected
NF	nuclear factor
NHPH	non-*Helicobacter pylori Helicobacter*
NLRP	nucleotide-binding oligomerization domain-like receptor family, pyrin domain containing 3
NOD	nucleotide-binding oligomerization domain
NSAID	non-steroidal anti-inflammatory drug
Ocln	Occluding

OHDA	Hydroxydopamine
OipA	outer inflammatory protein A
OMP	outer membrane protein
PAMP	pathogen-associated molecular patterns
PAR	pseudo-autosomal region
PARK	parkin gene
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PD	Parkinson's disease
PgbA/B	plasminogen-binding protein A/B
PH	measure of acidity or basicity
PINK	phosphatase and tensin homolog (PTEN)-induced putative kinase 1
PorA/B/C/D	pyruvate oxidoreductase complex
PPI	proton pump inhibitor
PRR	pattern recognition receptor
PrtC	gene for the production of collagenase
PTEN	phosphatase and tensin homolog
PutA/B	proline utilization A/B gene
RdxA	oxygen-insensitive NAD(P)H-nitroreductase gene
ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
RT	room temperature
RT-PCR	real-time PCR
RUT	rapid urease test
SabA	sialic acid binding adhesion A
SAT	stool antigen test
SodB	superoxide dismutase
SPF	specific pathogen free
Spp.	Species
SSCR	simple sequence cytosine repeat
T4SS	type IV secretion system
TEER	transepithelial electrical resistance
TGF	tissue growth factor
Th	T-helper cell
Tlp	methyl-accepting chemotaxis proteins
TLR	toll-like receptor
TNF	tumour necrosis factor
Treg	regulatory T cell

UBT	urea breath test
UreA/B	urease subunit A/B
VacA	vacuolating cytotoxin
VIP	vasoactive intestinal peptide
VIPR	vasoactive intestinal peptide receptor
ZO	zonulae occludentes

REVIEW OF THE LITERATURE

The present thesis deals with *Helicobacter suis* (*H. suis*), a bacterium that naturally colonizes the stomach of pigs and non-human primates. Infections with this microorganism have also been associated with gastric disease in humans.

Here, we first present an overview of the genus *Helicobacter* and the prevalence, phylogeny and clinical significance of gastric *Helicobacter* infections in humans. Then, the different diagnostic methods for detection of gastric *Helicobacter* infections are discussed. Additionally, the prevalence of *H. suis* in pigs and the pathogenic significance of this agent for pigs, non-human primates and experimentally infected lab animals are considered. Subsequently, more information is provided on the genomics and genetics as well as the host immune response against *H. suis* infections. However, since only limited information is available for *H. suis* on these two subjects, other gastric *Helicobacter* spp. and in particular *H. pylori,* are considered here as well. Finally, a brief overview is presented on the association between *H. pylori* infections and extragastric manifestations, with emphasis on Parkinson's disease (PD).

1. The rise of the genus *Helicobacter*

In 1984, Marshall and Warren reported the presence of a typical curved bacterium in patients with active chronic gastritis, duodenal or gastric ulcers and highlighted its importance in the etiology of these diseases (Marshall and Warren, 1984). Initially this microorganism was believed to be a member of the genus *Campylobacter*. However, major differences in ultrastructural features, cellular fatty acids and the *5S* and/or *16S rRNA* gene sequences suggested that this bacterium did not belong to the genus *Campylobacter* but to a novel genus. This microorganism was therefore designated *Helicobacter pylori* (Goodwin et al., 1989). Since the description of this human pathogen, many other species in the genus *Helicobacter* have been described. Several of those possess the ability to colonize animal hosts and some of them have a zoonotic potential in humans (Haesebrouck et al., 2009; Flahou et al., 2016). The *Helicobacter* bacteria can roughly be divided into gastric and enterohepatic species. Gastric *Helicobacter* species are able to survive the hostile, acidic environment in the stomach by expressing urease at a high level (Weeks et al., 2000; Pot et al., 2007). Enterohepatic helicobacters thrive on the mucosal surfaces of the intestinal tract and/or the liver (Sterzenbach et al., 2007). Currently the *Helicobacter* genus comprises 46 identified species. An overview of the different species, their hosts, disease associations and zoonotic potential is shown in **Table 1**.

Table 1: *Helicobacter* spp. and their pathogenic significance for humans and animals (adapted from Flahou et al., 2016).

Group	Taxon	Natural host	Disease associations	Zoonotic potential
gastric *Helicobacter* spp.	*'Candidatus* H. bovis'*	cattle	ND	yes
	'Candidatus H. hominineae'*	chimpanzee, gorilla	ND	unknown

	H. acinonychis	cheetah, tiger, lion	severe chronic gastritis	unknown
	H. ailurogastricus	cat	asymptomatic, gastritis, peptic ulcers, mucosa-associated lymphoid tissue lymphoma (MALT)-lymphoma	unknown
	H. baculiformis	cat	ND	unknown
	H. bizzozeronii	cat, dog	asymptomatic, gastritis, peptic ulcers, MALT-lymphoma	yes
	H. cetorum	whale, dolphin	asymptomatic, gastritis, peptic ulcers	unknown
	H. cynogastricus	dog	ND	unknown
	H. felis	dog, cat, cheetah, new guinea wild dog, rabbit	asymptomatic, gastritis, peptic ulcers, MALT-lymphoma	yes
	H. heilmannii	dog, cat, cheetah, bobcat, tiger, lynx, leopard, puma	asymptomatic, gastritis, peptic ulcers, MALT-lymphoma	yes
	H. mustelae	ferret	asymptomatic, gastritis, peptic ulcers, gastric cancer, MALT-lymphoma	unknown
	H. pylori	human	asymptomatic, gastritis, peptic ulcers, gastric cancer, MALT-lymphoma	/
	H. salomonis	dog, cat, rabbit	asymptomatic, gastritis, peptic ulcers, MALT-lymphoma	yes
	H. suis	pig, crabeating macaque, rhesus macaque, mandrill monkey	asymptomatic, gastritis, peptic ulcers, MALT-lymphoma	yes
enterohepatic *Helicobacter* spp.	'*Candidatus* H. colifelis'*	cat	ND	unknown
	H. anseris	goose	ND	unknown
	H. aurati	hamster	asymptomatic, hepatic and intestinal disease	unknown
	H. bilis	mouse, rat, Mongolian gerbil, dog, cat, sheep	asymptomatic, hepatic and intestinal disease	yes
	H. brantae	goose	ND	unknown
	H. callitrichis	marmoset	ND	unknown
	H. canadensis	bird, pig	ND	yes
	H. canis	dog, cat	asymptomatic, diarrhea, hepatitis, gastroenteritis	yes

	H. cholecystus	hamster	asymptomatic, hepatic and intestinal disease	unknown
	H. cinaedi	hamster, cat, dog, rat, rhesus monkey, baboon	asymptomatic, hepatic and intestinal disease	Yes
	H. equorum	horse	ND	Unknown
	H. fennelliae	dog	asymptomatic, enteritis, proctitis, proctocolitis	Yes
	H. ganmani	mouse	asymptomatic, hepatic and intestinal disease	Yes
	H. hepaticus	mouse, Mongolian gerbil	asymptomatic, hepatic and intestinal disease	Yes
	H. macacae	baboon, rhesus monkey	asymptomatic, chronic colitis, intestinal adenocarcinoma	Unknown
	H. marmotae	cat, woodchuck	asymptomatic, hepatitis, typhlocolitis	Unknown
	H. magdeburgensis	mouse	asymptomatic, hepatic and intestinal disease	Unknown
	H. mastomyrinus	rodents	asymptomatic, hepatic and intestinal disease	Unknown
	H. mesocricetorum	hamster	asymptomatic, hepatic and intestinal disease	Unknown
	H. muricola	mouse	asymptomatic, hepatic and intestinal disease	Unknown
	H. muridarum	rat, mouse	asymptomatic, hepatic and intestinal disease	Unknown
	H. pamatensis	pig, cat, bird	ND	Yes
	H. pullorum	poultry	asymptomatic, hepatitis, diarrhea, enteritis	Yes
	H. rappini	sheep, dog, mouse	ND	Yes
	H. rodentium	rat, mouse	asymptomatic, hepatic and intestinal disease	Unknown
	H. saguini	cotton-top tamarin	asymptomatic, ulcerative colitis, typhlocolitis and dysplasia	Unknown
	H. suncus	house musk shrew	ND	Unknown
	H. trogontum	sheep, pig, mouse	asymptomatic, hepatic and intestinal disease	Unknown
	H. typhlonius	rat, mouse	asymptomatic, hepatic and intestinal disease	Unknown

	H. valdiviensis	wild birds	ND	Unknown
	H. westmeadii	human	asymptomatic, inflammatory bowel disease	/
	H. winghamensis	human, wild rodents	asymptomatic, gastroenteritis	Yes

ND: Not described
*: *Helicobacter* species that has not yet been isolated and cultivated *in vitro*

2. Gastric *Helicobacter* species in humans with emphasis on zoonotic pathogens

H. pylori is the best studied and most prevalent of the *Helicobacter* species which colonize the human stomach. Infections with this microorganism have not only been associated with gastritis and peptic ulcer disease as described above, but also with more severe gastric pathologies including mucosa-associated lymphoid tissue (MALT-) lymphoma and adenocarcinoma (Stolte et al., 1993; Kusters et al., 2006). *H. pylori* infections are frequently acquired during early childhood and the disease progresses with age (Suerbaum & Josenhans, 2007). Most of the *H. pylori* infections remain asymptomatic. Only 10-20% of the people carrying *H. pylori* will develop gastric disorders (Kusters et al., 2006; Suerbaum and Josenhans, 2007). **Figure 1** provides an overview of the different *H. pylori*-associated gastric disease outcomes in human patients.

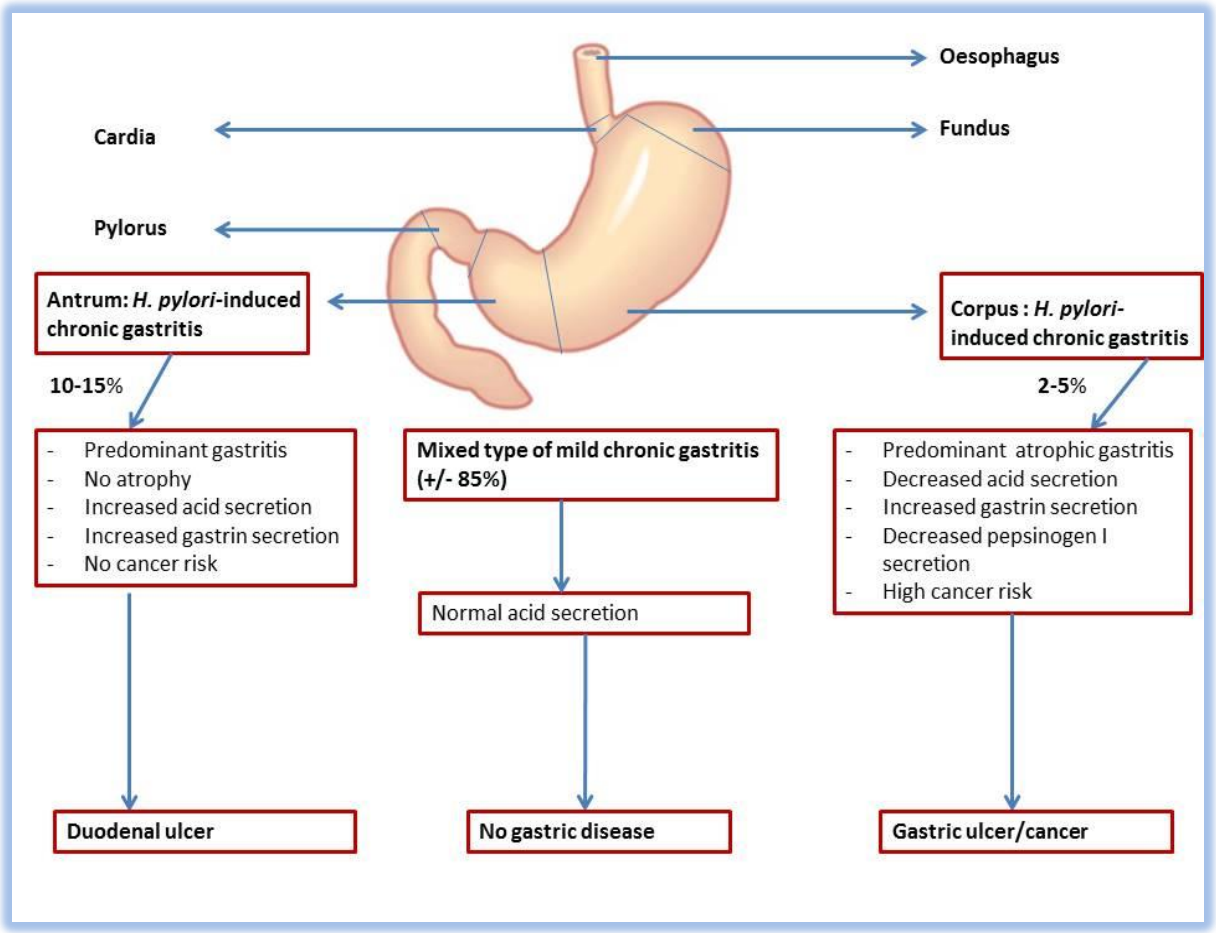

Figure 1: Sequential steps of gastric disease resulting from *H. pylori* infection**.** Depending on the localization of the *H. pylori*-induced chronic gastritis, no gastric disease, duodenal ulcers, gastric ulcer disease or cancer may develop. In 85% of the patients, an infection with *H. pylori* will not result in the development of gastric diseases. In 10-15% of the *H. pylori*-infected patients, gastritis is mainly localized in the antrum (antrum predominant gastritis), which results in increased acid secretion

and successively in the development of duodenal ulcers. In only 2-5% of the patients, *H. pylori* infection will lead to the development of atrophic gastritis in the corpus of the stomach. This latter pathology is accompanied by decreased acid secretion as a result of atrophy of the acid producing glands, thereby increasing the risk to develop gastric cancer. The precancerous cascade progresses from multifocal atrophic gastritis, through intestinal metaplasia, to dysplasia, the severe grade represents carcinoma *in situ*, then invasive adenocarcinoma. Chronic gastritis is associated with MALT-lymphoma. Gastric ulcer occurs with corpus gastritis, before the stage of achlorhydria. Depending on the virulence of the *H. pylori* strain, the environmental factors and the host immune response, infections with this bacterium will or will not result in the development of gastric adenocarcinoma (Karczewska et al., 2014).

It is estimated that half of the human population in the world is infected with *H. pylori* albeit with large geographical variations. In developing countries, more than 80% of the human population is infected with *H. pylori*, whereas the prevalence rate of *H. pylori* in developed countries varies from 20 to 50% (Kusters et al., 2006; Pounder and Ng, 1995). This drop in prevalence in the Western world may create a niche for colonization of the human stomach by other organisms (Pounder and Ng 1995; Kusters et al., 2006).

Indeed, gastric disease in humans has also been associated with other, long, spiral-shaped bacteria. These gastric non-*H. pylori Helicobacter* spp. (NHPH) are similar in morphology to bacteria earlier reported in the stomach of pigs, cats, dogs and non-human primates (Salomon, 1898; Mendes et al., 1990; Queiroz et al., 1990). Generally, they have been found in 0.2 to 6% of gastric biopsies from humans with severe gastric complaints (Heilmann and Borchard, 1991; Stolte et al., 1994; Svec et al., 2000; Solnick et al., 2003). NHPH include *H. suis* from pigs and non-human primates and *H. felis, H. bizzozeronii, H. salomonis* and *H. heilmannii* from cats and dogs (Haesebrouck et al., 2009; Flahou et al., 2016). Although *H. cynogastricus*, *H. baculiformis* and *H. ailurogastricus* also have pets as natural host, their zoonotic significance currently remains unknown. Besides these long spiral-shaped helicobacters, 3 other animal-associated gastric *Helicobacter* spp., with a slightly-curved morphology more similar to that of *H. pylori*, are known: *H. acinonychis* from wild large felines, *H. cetorum* from marine mammals and *H. mustelae* from ferrets (Paster et al., 1991; Harper et al., 2002; Eaton et al., 1993). Infections of humans with those agents have not been described.

2.1 Phylogeny of animal-associated gastric *Helicobacter* species

Although minor differences in morphology among the porcine, feline and canine gastric NHPH have been described (Haesebrouck et al., 2009, Smet et al., 2011; Joosten et al., 2015), species identification based on microscopic analysis of human biopsy samples is not recommended (Eaton et al., 1996). Therefore, analysis of the *Helicobacter 16S rRNA* gene sequence has frequently been used in the past for differentiation between gastric *Helicobacter* species (O'Rourke et al., 2004b). However, sequencing of the *16S rRNA* gene cannot distinguish between the canine and feline helicobacters, including *H. bizzozeronii*, *H. felis*, *H. salomonis*, *H. baculiformis*, *H. cynogastricus* and *H. heilmannii* (Smet et al., 2011; Baele et al., 2008a; Van den Bulck et al., 2006; Dewhirst et al., 2005). For differentiation between those species, analyses of the *ureA* and *ureB* and the *hsp60* gene sequences have been performed. The similarity of their *ureAB* genes is lower than 85%, which is sufficient for discrimination between these species (O'Rourke et al., 2004b). It has recently been shown that the newly described *H. ailurogastricus* species from cats can not be distinguished from its closest relative *H. heilmannii* by means of its *16S rRNA* and *ureAB* genes but by comparison of their complete genome sequences

(Joosten et al., 2015). This means that the discriminatory capacity of these gene sequences is not high enough for the distinction of closely related gastric *Helicobacter* species. In this respect, genome sequencing based approaches are superior compared to the traditional *16S rRNA* sequence analysis for studying phylogeny because they are based on the complete genome content and have a better resolution for discriminating both distantly and closely related bacteria (Vandamme, 2014). **Figure 2** is an illustration of a phylogenetic tree based on the core genomes of all gastric helicobacters from which the genome sequence is publically available (Joosten et al., 2015).

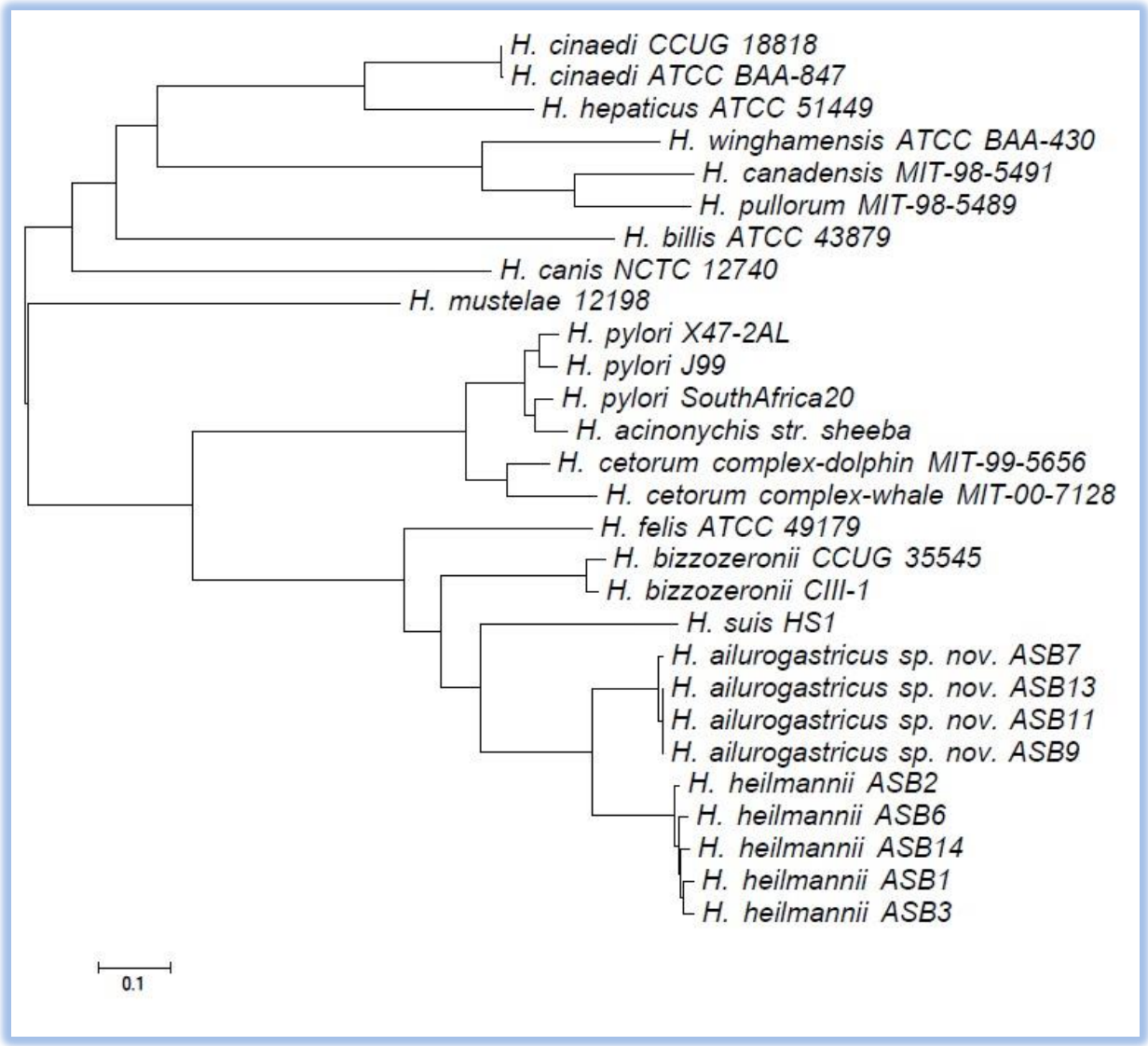

Figure 2: Phylogenetic tree based on the aligned and concatenated core genes of *Helicobacter* species from which the genomic content is known. The phylogram represents a maximum-likelyhood tree of gastric and enterohepatic *Helicobacter* spp. based on 303 aligned and concatenated core genes. The enterohepatic *Helicobacter* spp. (*H. cinaedi*, *H. hepaticus*, *H. winghamensis*, *H. canadensis*, *H. pullorum*, *H. billis*, *H. canis*) were used as an outgroup. Approximate likelihood ratio test values of >99% were obtained for all nodes. Optimization of topology, branch length and parameters of the starting tree were performed (adapted from Joosten et al., 2015).

2.2 Clinical significance of gastric non-*Helicobacter pylori Helicobacter* infections in humans

A number of reports have highlighted that people having close contact with dogs, cats and pigs are more likely to be infected with an animal-associated gastric *Helicobacter* species (Chung et al., 2014; De Bock et al., 2007; Joosten et al., 2013; Meining et al., 1998). *Helicobacter* DNA has been detected in saliva from cats, dogs and pigs, indicating that the oral cavity of these animals may act as source of NHPH infections for humans (Ekman et al., 2013; Casagrande Proieti et al., 2010; ShojaeeTabrizi et al., 2010). Fecal-oral transmission has also been suggested as a possible route for infection in cats (Ghil et al., 2009). Besides direct contact with animals, other routes of transmission of NHPH should not be neglected. It has been shown that *H. felis* is able to survive in water for several days highlighting the possible role for water in the transmission of this species as well (Azevedo et al., 2008). *H. suis* can be present on and survive in minced pork indicating that raw or undercooked pork may also constitute a source of *H. suis* infection for humans (De Cooman et al., 2013).

H. suis, *H. heilmannii*, *H. bizzozeronii*, *H. felis* and *H. salomonis* (**Table 1**) have been associated with gastritis, gastric and duodenal ulcers and low grade MALT-lymphoma in humans (Haesebrouck et al., 2009; Matsumoto et al., 2014; Iwanczak et al., 2012; Duquenoy and Le Luyer, 2009). They have not been associated so far with gastric adenocarcinoma. The gastritis induced by NHPH is usually less active and less severe compared to *H. pylori* but the risk to develop MALT-lymphoma is higher (Morgner et al., 2000; Haesebrouck et al., 2009). Clinical signs associated with NHPH infections in humans include: epigastric pain, nausea, hematemesis, irregular defecation frequency and consistency, vomiting, recurrent dyspepsia, heartburn, dysphagia and decreased appetite (Dieterich et al., 1998; Goddard et al., 1997; Heilmann and Borchard, 1991; Kaklikkaya et al., 2002; Mention et al., 1999; Oliva et al., 1993; Schildt et al., 2000; Seo et al., 2003; Sykora et al., 2003; Van Loon et al., 2003; Wegmann et al., 1991; Yang et al., 1998; Yoshimura et al., 2002). In patients undergoing endoscopy, a variety of lesions can be observed ranging from a normal to slightly hyperaemic mucosa, mucosal oedema, nodular inflammation and the presence of ulcerations in the antrum of the stomach or in the duodenum (Haesebrouck et al., 2009; Sykora et al., 2003; Van Loon et al., 2003; Yang et al., 1995; Yoshimura et al., 2002). Histological examination of gastric biopsies generally reveals infiltration of lymphocytes and plasma cells. In some cases, lymphocytic aggregates or intestinal metaplasia have been described (Ierardi et al., 2001; Joosten et al., 2013; Matsumoto et al., 2014; Morgner et al., 2000; Yakoob et al., 2012).

In several studies the prevalence of gastric NHPH was investigated by analyzing human biopsies from patients with gastric complaints (Trebesius et al., 2001; De Groote et al., 2005; Van den Bulck et al., 2005). In general, NHPH have been detected in 0.2–6% of these patients, with *H. suis* being the most prevalent NHPH species (Haesebrouck et al., 2009).

Treatment of gastric *Helicobacter* infections is recommended in case of *Helicobacter*-related symptoms or in order to prevent the evolution to atrophic gastritis and gastric cancer (Cats et al., 1998; Kusters and Kuipers, 2001). Like for *H. pylori* eradication, the treatment of a NHPH infection consists of the administration of a proton pump inhibitor (PPI) in combination with two antibiotics, such as clarithromycin and tetracycline or amoxicillin. However, failure of successful eradication of both *H. pylori* and NHPH infections has been reported

and may be due to an increasing occurrence of antimicrobial resistance (Kivisto et al., 2010; Megraud et al., 2004; Horiki et al., 2008; Graham et al., 2010). An overview of what is known so far for NHPH is given below.

Van den Bulck et al. (2005) studied the susceptibilities of animal-derived *H. felis*, *H. bizzozeronii*, and *H. salomonis* isolates to 10 antimicrobial agents. All species were highly susceptible to ampicillin, clarithromycin, tetracycline, tylosin, enrofloxacin, gentamicin, and neomycin. This was demonstrated by low Minimum Inhibitory Concentrations (MICs). Higher MICs were obtained for lincomycin and spectinomycin. Two *H. felis* strains showed a MIC of 16 µg/ml for metronidazole, suggesting acquired resistance to this antimicrobial agent (Van den Bulck et al., 2005). Another study highlighted that *H. bizzozeronii* and potentially other NHPH easily acquire clinically relevant resistance to metronidazole due to the high mutation rate of the simple sequence cytosine repeat (SSCR) located in the 3' region of the oxygen-insensitive NAD(P)H-nitroreductase (*rdxA*) gene (Kondadi et al., 2013). For this reason, metronidazole, lincomycin and spectinomycin are considered as second line therapy. Vermoote et al. (2011) reported the presence of acquired resistance to fluoroquinolones in a porcine *H. suis* isolate. For all tested *H. suis* strains, the MIC of ampicillin was higher than described for *H. felis, H. bizzozeronii, H. salomonis* and *H. pylori,* possibly indicating intrinsic reduced susceptibility of *H. suis* to aminopenicillins compared to other gastric *Helicobacter* species. The significance of this finding for the treatment of *H. suis* infections is currently not known. However there is a report of a patient with a spiral gastric *Helicobacter* infection, which relapsed after triple therapy including amoxicillin and clarithromycin, and was then eradicated by quadruple therapy containing tetracycline and bismuth (Dobbs et al 2005). This organism was reported to be *H. heilmannii* by 16S rRNA based PCR, but was later shown to be *H. suis*.

2.3 Diagnosis of gastric *Helicobacter* infections

To date, several non-invasive and invasive diagnostic tools are available for the detection of gastric infections with *Helicobacter* in general and *H. pylori* in particular. An overview is given in **Table 2** and the most frequently used tests are described below in more detail.

Table 2: Overview of the available non- or minor invasive diagnostic tests for the identification of gastric *Helicobacter* spp.

Diagnostic test	Type	Identification	Advantages	Disadvantages	References
urea breath test	non-invasive	*H. pylori*	- simple and safe - high sensitivity/specificity in adults - initial screening - confirmation eradication	- variable sensitivity/specificity in children - variable sensitivity/specificity in case of partial gastrectomy and peptic ulcer bleeding	Malfertheiner et al., 2012; Guarner et al., 2010 ; Barkun et al., 2010 ; Velayos et al., 2012 ; Wardi et al., 2012
		gastric NHPH species	- simple and safe - initial screening	- false negative results - difficult use in animals - test results depending on colonization, speed of gastric emptying and recent use of antibiotics	Okiyama et al., 2005; Matsui et al., 2014 ; Blaecher et al.,2013 ; Cornetta et al., 1998
stool antigen test (SAT)	non-invasive	*H. pylori*	- safe, fast and reliable - use in children	- variable accuracy - reduced sensitivity in	Gisbert and Pajares, 2006; Saez et al., 2012;

			- use after distal gastrectomy	gastrointestinal bleeding - reduced accuracy in unformed or watery stool samples	Cardenas et al., 2008; Quieroz et al., 2013 ; Adamopoulos et al., 2009 ; Yan et al., 2010
		gastric NHPH species		- not available	
serology	non-invasive	*H. pylori*	- use for initial screening, patients using antibiotics, patients suffering from acute bleeding	- variability in sensitivity and specificity - variability in efficiency depending on *H. pylori* strain (heterogeneity between strains), geographical area and host genetic differences - no discrimination between past and present infection	Rubicz et al., 2011; Mégraud et al., 2012 ; Burucoa et al., 2013; Vale et al., 2008-2009 ; Vitoriano et al., 2011 ; Malfertheiner et al., 2007 ; Hirschl and Rotter, 1996 ; Choi et al., 2011 ; Herbrink and van Doorn, 2000
		gastric NHPH species		- not available	
enzyme-linked immunosorbent assay (ELISA)	non-invasive	*H. pylori*	- easy and reliable - rather high sensitivity and specificity - use for saliva and dental plaques	- use in young children (development adaptive immune system)	Bode et al., 2002; Raymond et al., 1996 ; Luzza et al., 1997 ; De Pascalis et al., 1999 ; Sunnerstam et al., 1999 ; Malaty et al., 2000
		gastric NHPH species		- not available	
polymerase chain reaction (PCR)	non-invasive	*H. pylori*	- use for fecal samples - use for dental plaques, saliva	- fecal samples: false negative results as a result of food degradation products, PCR- inhibitory factors and overload of bacterial species	Teoman et al., 2007; Souto and Colombo, 2008
		gastric NHPH species	- fecal samples: decent image on the presence of enterohepatic *Helicobacter* spp.	- fecal samples: false negative results as a result of food degradation products, PCR- inhibitory factors and overload of bacterial species	Shinozaki et al., 2002
	Invasive	*H. pylori*	- identification and quantification in gastric biopsies - high sensitivity and specificity -identification of virulence factors	- variation as a result of transport conditions and number of specimens	Qin et al., 2016; Schabereiter-Gurtner et al., 2004; Ndip et al., 2003; Ricci et al., 2007; De Martel et al., 2010
		gastric NHPH species	- identification and quantification in gastric biopsies - high sensitivity and specificity - differentiation between gastric NHPH species - strain differentiation	- variation as a result of transport conditions and number of specimens -aspecific interactions with host DNA and DNA from other NHPH species	Dieterich et al., 1998; De Groote et al., 2001; Hwang et al., 2002; Baele et al., 2004; O'Rourke et al., 2004; Trebesius et al., 2001; Matsumoto et al., 2009; Kivisto et al., 2010; Wuppenhorst et al., 2013; Joosten et al., 2013; Liang et al., 2013; Matsui et al., 2014
endoscopy	Invasive	*H. pylori* and gastric NHPH species	- visualization of macroscopic abnormalities in the stomach	- not reliable - low sensitivity and specificity - no differentiation between gastric *Helicobacter* spp.	Haesebrouck et al., 2009; Kato et al., 2013; Oguri et al., 2007; Watanabe et al., 2012; Kusters et al., 2006;

rapid urease test (RUT)	Invasive	*H. pylori* and gastric NHPH species	- relatively high specificity and sensitivity - low cost and rapid diagnosis - semi-quantitative	- lower sensitivity in case of: peptic ulcer bleeding, partial gastrectomy and formalin contamination of forceps - false negatives in case of low grade colonization	Tseng et al., 2005; Gisbert et al., 2006; Ozaslan et al., 2010; Choi et al., 2012; Tian et al., 2012; Happonen et al., 1998
histology	Invasive	*H. pylori*	- evaluation of status gastric mucosa - cheap versus expensive staining	- reliability depends on localization and number of gastric biopsies - variability as a result of use of proton pump inhibitors, leading to false negative results	Wang et al., 2010; Aggarwal et al., 2011; El-Zimaity et al., 2013; Lash and Genta et al., 2013
		gastric NHPH species	- evaluation of status gastric mucosa - cheap versus expensive staining	- detection hampered by focal distribution of bacteria - not recommended for identification up to species level	Wang et al., 2010; Aggarwal et al., 2011; Lash and Genta et al., 2013; Happonen et al., 1998; Stoffel et al., 2000
isolation and cultivation	Invasive	*H. pylori*	- golden standard -specificity up to 100%	- variability in sensitivity depending on quality, quantity and transport conditions of gastric biopsies.	Ndip et al., 2003; Ricci et al., 2007; Kusters et al., 2006
		gastric NHPH species	-specificity up to 100%	- low sensitivity - low success rate - laborious procedure	Ndip et al., 2003; Ricci et al., 2007; Kusters et al., 2006; Happonen et al., 1998; Javala et al., 1998; Haesebrouck et al., 2009

2.3.1 Non- or minor invasive tests

2.3.1.1 The urea breath test

A frequently used test for the detection of gastric *Helicobacter* species is the urea breath test. This simple and safe non-invasive test has been reported to be highly sensitive (88%-95%) and specific (95%-100%) for the detection of *H. pylori* in adults, whereas in children a large variability in sensitivity and specificity, ranging from 75% up to 100% has been observed (Malfertheiner et al., 2012; Guarner et al., 2010). Because of the rather low sensitivity and specificity, the reliability of the urea breath test has likewise been questioned when applied after emergency endoscopy in peptic ulcer bleeding and in case of partial gastrectomy (Barkun et al., 2010; Velayos et al., 2012; Wardy et al., 2012). The principle of the urea breath test is based on the urease activity of gastric *Helicobacter* species. The presence of urease in the stomach breaks down the orally administered ^{13}C-labeled or radioactive ^{14}C-labeled urea into ammonia and soluble $^{13/14}CO_2$, which is absorbed by the gastric mucosa, transported by the systemic circulation and excreted in the exhaled breath. Finally the amount of exhaled $^{13/14}C$ is calculated by measurement of the ratio of $^{13/14}C$ to ^{12}C in the exhaled breath or the serum of the patient (Logan, 1998; Savarino et al. 1999; Graham and Klein, 2000; Gisbert and Pajares, 2004). An increase over baseline, 30 minutes after urea ingestion, more than a specified value constitutes a positive result. In general, the use of the urea breath test is recommended in case of initial diagnosis of *Helicobacter* and for confirmation of *Helicobacter* eradication after treatment (Malfertheiner et al., 2012). Up to date, limited information is available on the sensitivity and specificity of the urea breath test for the detection of NHPH

infections in humans. Although all gastric NHPH species are characterized by their urease activity (Haesebrouck et al., 2009), false negative results are often obtained when the urea breath test is performed in NHPH-infected human patients (Okiyama et al.. 2005; Matsui et al., 2014). Possible explanations might be the patchy distribution of these microorganisms in the human stomach (Haesebrouck et al., 2009) and the relative low colonization density (Matsui et al., 2014).

2.3.1.2 Stool antigen test (SAT)

The stool antigen test (SAT) is a non-invasive diagnostic tool based on the detection of *H. pylori* antigens in human fecal samples (Gisbert and Pajares, 2006). This test is frequently applied for the screening of *H. pylori* in children (Cirak et al., 2007; Choi et al., 2011; Chehter et al., 2013). Although SATs are considered to be safe, fast and reliable for the identification of *H. pylori* infections (Gisbert et Pajares, 2006), the accuracy is influenced by antigenicity of the *H. pylori* strains as well as by the time between sample collection and measurements (Ritchie et al., 2009; Queiroz et al., 2013). A decreased sensitivity is found in patients suffering from gastrointestinal bleeding, whereas unformed or watery stool samples can result in a reduced accuracy because of the diluting effect on the *H. pylori* antigens (Saez et al., 2012). Two types of highly sensitive and specific SATs are available: the immunochromatography assay (ICA) and the enzyme immune assay (EIA). As ICA-tests are easy to apply and do not require any specialized equipment, they are frequently used for rapid diagnosis (Shibayama et al., 2011). They are, however, less reliable than the EIA- tests (Gisbert et al., 2006; Shibayama et al., 2011; Sato et al., 2012; Korkmaz et al., 2013). The ICA tests are often presented as a test-strip (eg. Testmate rapid pylori antigen). The presence of *H. pylori* antigens in a suspended stool sample results in the formation of immune complexes with color-labeled monoclonal antibodies. Migration of these antigen-antibody complexes over the surface of strip results in binding with secondary antibodies, which is visualized by the appearance of a colored line on the test-strip (Suzuki et al., 2002). In EIA tests the optical density is measured after incubation and washing of a mixture of suspended stool and peroxidase-conjugated *H. pylori* antibodies (Suzuki et al., 2002). Except for controversial results in post-eradication assessment, EIAs based on polyclonal antibodies are reliable diagnostic tests for the detection of *H. pylori* infections (Odaka et al., 2002; Veijola et al., 2005). Nevertheless, monoclonal antibody based SATs generally represent a higher specificity compared to polyclonal based SATs (Dominguez et al., 2006; Deguchi et al., 2009) and they are considered more reliable than the urea breath test in children (Cardenas et al., 2008; Queiroz et al., 2013) and in patients who underwent distal gastrectomy (Adamopoulos et al., 2009; Yan et al., 2010). Currently, no antigen assays have been developed for the detection of gastric NHPH infections in human patients.

2.3.2 Invasive tests

2.3.2.1 Endoscopy and analysis of gastric biopsies

Endoscopy is frequently performed to evaluate the gastric mucosa for the presence of gross abnormalities. Endoscopic findings characteristic for an *H. pylori* infection are: diffuse redness, spotted redness, mucosal swelling, erosions and peptic ulcers (Haesebrouck et al., 2009; Kato et al., 2013). Diagnosis of *Helicobacter*

infections based solely on endoscopic findings is not reliable enough because of the low sensitivity and specificity. In order to obtain a more trustworthy diagnosis, gastric biopsy samples need to be further examined using histology, PCR techniques and/or *in vitro* cultivation. Biopsy is, of course, needed for gastric RUT. Here the biopsy is placed in a chamber containing urea and an indicator, which changes colour in presence of ammonia. Several studies have indicated that the corpus greater curve, in general, is the most optimal biopsy site for the diagnosis of *H. pylori* infections (Calvet et al. 2013; El-Zimaity et al., 2013). It may not be appropriate in antral or pan gastritis.

2.3.2.1.1 Histology

In general, histological examination of gastric biopsies is considered as a standard method for the detection of an *H. pylori* infection. However, the reliability depends on the location of biopsy collection in the stomach and the number of biopsies (El-Zimaity et al., 2013). Histology has the advantage that additional information can be obtained on the status of the gastric mucosa, such as the presence of acute or chronic inflammation, lymphoid aggregates, intestinal metaplasia and glandular atrophy (Lash and Genta, 2013).

For identification of *H. pylori,* the haematoxylin eosin (HE) staining is, in terms of cost-effectiveness, the most designated staining (**Figure 3**) (Wang et al., 2010). Other stainings that have been applied for the detection of *H. pylori* in gastric biopsies, brush cytology and impression smear cytology specimens are: Warthin-Starry-staining, Gram-staining, Genta-staining, modified Giemsa-staining, toluidine-blue-staining and Romanowski-staining (Warren and Marshall, 1983; Mendoza et al. 1993; Mirsa et al., 1993). Although *H. pylori* visualization is also possible using immunohistochemical stainings, application of these specialized, more expensive stainings is only justified for analysis of biopsies derived from patients with chronic active and inactive gastritis and for biopsies collected to confirm *Helicobacter* eradication therapy (Wang et al., 2010; Aggarwal et al., 2011). Like for *H. pylori,* diagnosis of infections with other gastric *Helicobacter* species in human biopsies is also routinely performed by histology and cytology (Debongnie et al. 1995). However, detection of NHPH infections is hampered by the focal distribution of these bacteria and therefore infection with these agents is often missed (Haesebrouck et al., 2009).

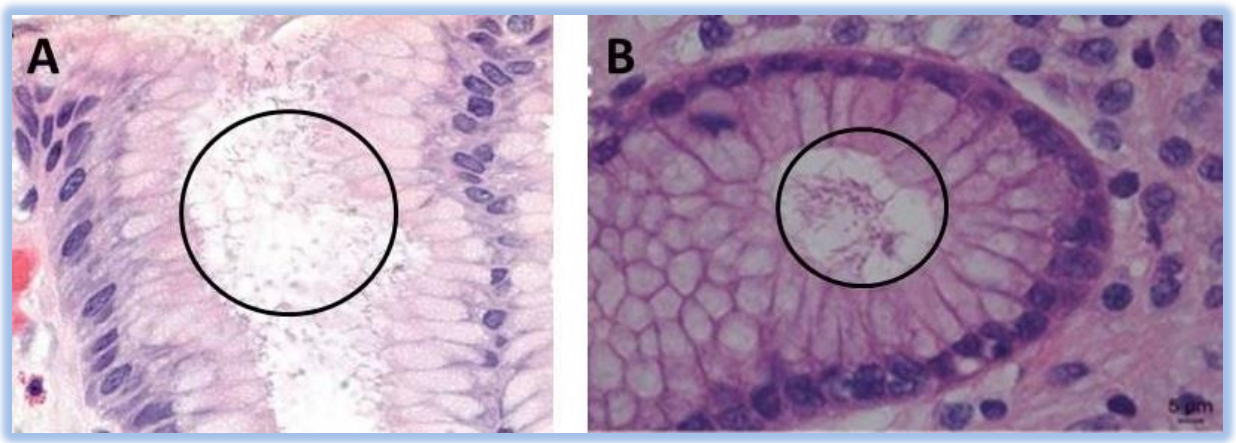

Figure 3: HE staining of the gastric mucosa of *Helicobacter*-infected human patients. (**A**) HE staining of the gastric mucosa of a *H. pylori*-infected human patient. The slightly curved *H. pylori* bacteria, visualized within the black circle, are mainly

located at the surface of the gastric epithelial cells or in the mucus layer in the stomach (adapted from Batts et al., 2013), (**B**) HE staining of the gastric mucosa of a *H. suis*-infected human patient. These spiral-shaped bacteria (within the black circle) are more frequently found in the gastric pits (adapted from Joosten et al., 2013).

2.3.2.1.2 Polymerase chain reaction

The polymerase chain reaction (PCR) can be used as a diagnostic tool to screen gastric biopsies for the presence of *Helicobacter* DNA. Moreover, using real-time (RT)-PCR, quantitation of bacteria in gastric biopsies is possible as well. In *H. pylori*-positive patients with severe precancerous lesions, detection of the vacuolating cytotoxin (*VacA*) gene in gastric biopsies using PCR has been proven really valuable (Schabereiter-Gurtner et al., 2004). PCR assays for the detection of gastric NHPH species are mainly based on the amplification of a part of the *16S rRNA* gene or the *ureA/B* genes (De Groote et al., 2001; Hwang et al., 2002; Baele et al., 2004; O'Rourke et al., 2004). Recently, a study was published describing the development of a PCR based on the *H. suis*-specific *carR* gene for identification of *H. suis* infections (Matsui et al., 2014). Despite the development of different PCR assays for the detection of NHPH in general and *H. suis* in particular, no information on their sensitivity and specificity is available.

2.3.2.1.3 Culture

Another method for the identification of gastric *Helicobacter* infections by analyses of gastric biopsies is isolation and cultivation of *Helicobacter*. Generally, *Helicobacter* grows under micro-aerobic conditions (5% O_2, 10% CO_2 and 85% N_2) at 37°C and at a pH ranging between 5.5 and 8 (Scott et al., 2002; Stingl et al., 2002; Kusters et al., 2006; Haesebrouck et al., 2009). The specificity for the detection of *H. pylori* infections by cultivation reaches up to 100% (Ndip et al., 2003), whereas significant variability in sensitivity has been described, depending on the quality, the quantity and the transportation conditions of the gastric biopsies (Ricci et al., 2007). Because of the high specificity and relative high sensitivity, cultivation is considered the golden standard for the diagnosis of *H. pylori* infections (Kusters et al., 2006). Compared to *H. pylori,* isolation and cultivation of gastric NHPH species has a low success rate due to the very fastidious nature of these bacteria (Happonen et al., 1998; Javala et al., 1998). So far, only *H. felis* (Wüppenhorst et al., 2012) and *H. bizzozeronii* (Andersen et al., 1999; Kivistö et al., 2010; Schott et al., 2011) have successfully been isolated from the human stomach.

2.3.3 Conclusion

Apart from histology, no commercial tests are currently available for the diagnosis of infections with NHPH in general and *H. suis* in particular. Therefore the development of accurate and reliable diagnostic techniques for the identification of gastric NHPH infections is required to obtain a credible picture on the prevalence of NHPH in humans.

3. *H. suis*: a porcine pathogen with a zoonotic potential for humans

3.1 Prevalence of *H. suis* in pigs

In general, a prevalence rate of 60% has been described for *H. suis* infections in pigs at slaughter age in Europe, Asia and South- and North-America (Barbosa et al., 1995; Grasso et al., 1996; Cantet et al., 1999; Roosendaal et al., 2000; Choi et al., 2001; Hellemans et al., 2007; Kopta et al., 2010). It has been suggested that *H. suis* is transmitted between pigs via the oral-oral route through saliva or via the gastric-oral route through vomiting or regurgitation, but this requires further investigation (Haesebrouck et al., 2009; Flahou et al., 2016).

A very high prevalence has been reported in adult pigs (> 80%). Lower infection rates have been shown in younger animals ranging from 2% in suckling piglets (Hellemans et al., 2007), 25 % in piglets after weaning (Hellemans et al., 2007) and 76 % in slaughter pigs of approximately 6 months old (Bosschem et al., 2016). This remarkable increase in prevalence of *H. suis* after weaning may suggest the presence of protective antibodies in the sow milk (Melnichouk et al., 1999; Hellemans et al., 2007). The protective effect of maternal milk against the colonization of *H. pylori* in the human and mouse stomach has also been described (Corthésy-Theulaz et al., 2003; Bhuiyan et al., 2010). In young piglets and animals at slaughter age, *H. suis* is mainly localized in the antrum and the pylorus region of the stomach, whereas this bacterium is mainly located in the fundus of the stomach in adult pigs (Hellemans et al., 2007; De Bruyne et al., 2012).

3.2 Pathogenic significance of *H. suis* in its natural hosts and experimentally infected laboratory animals

3.2.1 *H. suis* infections in pigs

Experimental and natural *H. suis* infections in pigs have been associated with chronic gastritis in the antrum of the stomach. Inflammation is mainly characterized by the infiltration of lymphoid cells and the development of lymphoid follicles (Mendes et al., 1991, Grasso et al., 1996, Queiroz et al., 1996; Park et al., 2000; Hellemans et al., 2007; De Bruyne et al., 2012). Besides the strong association with gastritis, infection with *H. suis* has also been associated with ulceration of pars oesophagea of the stomach (**Figure 4**) (Barbosa et al., 1995; Queiroz et al., 1996; Roosendaal et al., 2000; Choi et al., 2001), although *H. suis* does not colonize this area of the stomach. The exact role of *H. suis* in the development of this pathology remains to be further elucidated. *H. suis* infection in pigs has been associated with an increased number of gastrin producing cells and a decreased number of somatostatin producing cells in the antrum of the stomach (Sapierzynski et al., 2007). Since gastrin stimulates and somatostatin inhibits the secretion of hydrochloric acid by parietal cells, this may influence gastric acid production, which may also be altered due to the tropism of this bacterium for parietal cells (Hellemans et al., 2007). It has been suggested that such an altered gastric acid secretion might be involved in the development of ulceration of the pars oesophagea (Sapierzynski et al., 2007; Haesebrouck et al., 2009). Other research groups could not find a link between a *H. suis* infection and ulceration of the pars oesophagea (Grasso et al., 1996; Melnichouk et al., 1999; Park et al., 2000; Szeredi et al., 2005). Differences in virulence between *H. suis* strains, different sampling and laboratory techniques might explain these contradictory results.

Besides *H. suis,* other factors, such as particle size of the feed, the presence of short fatty acids in the stomach, the occurrence of concurrent diseases or changes in the microbiome, may also contribute to the development of gastric ulceration (**Figure 4**) (Hessing et al., 1992; Argenzio et al., 1996; Ayles et al., 1996; Krakowka et al., 1998; Lindberg et al., 2001; Robertson et al., 2002; Amory et al., 2006; Miller et al., 2012). The presence of ulcers in the porcine non-glandular mucosa of the stomach may result in decreased feed intake, a decrease in daily weight gain and even sudden death due to fatal hemorrhage (Ayles et al. 1996; Haesebrouck et al. 2009), thus leading to significant economic losses (**Figure 4**). A decrease in daily weight gain of up to 10 % has been observed in weaned piglets (6-8 weeks old) after 4 and 6 weeks of experimental infection with *H. suis,* although without a clear association with the development of ulceration in the non-glandular part (De Bruyne et al., 2012).

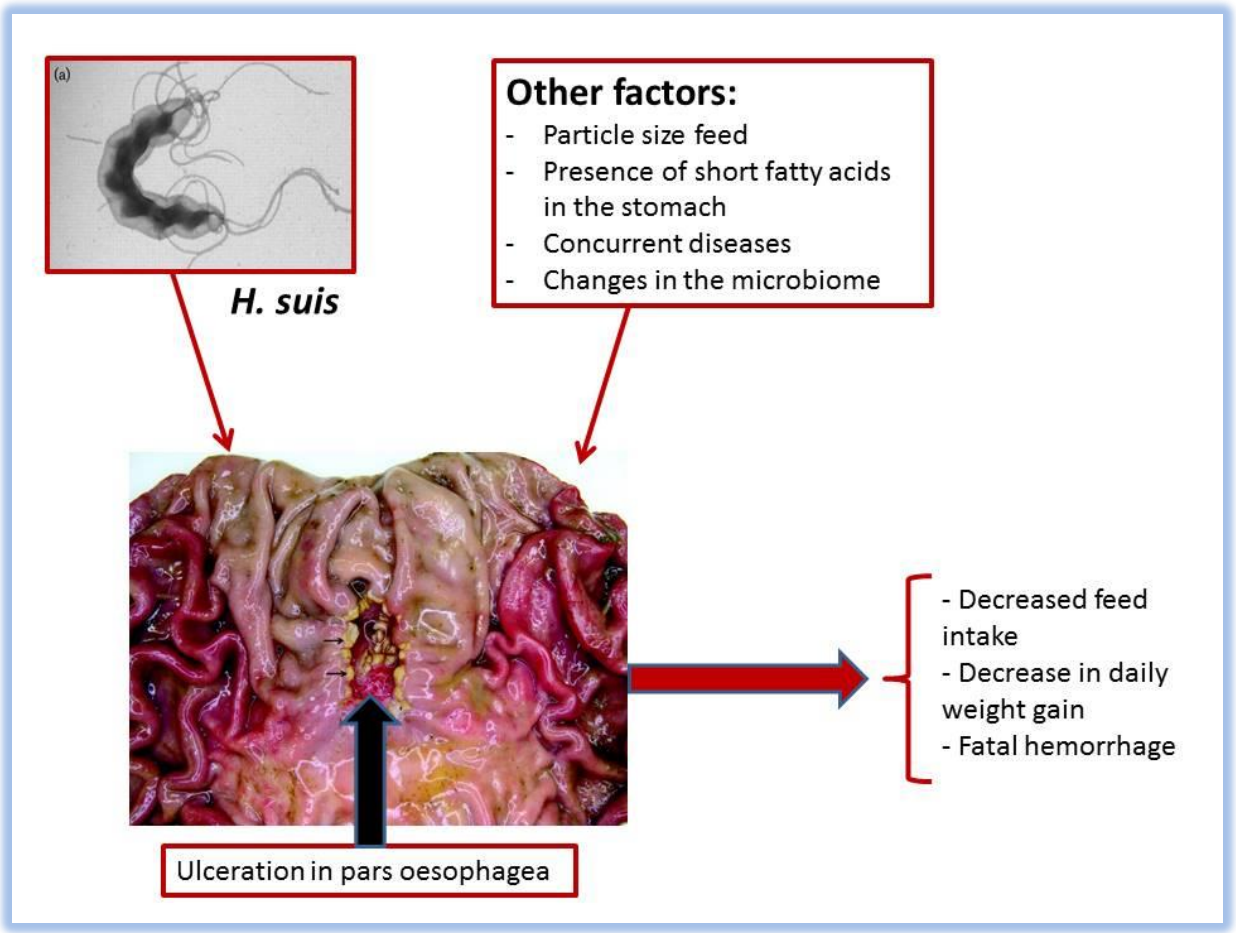

Figure 4: Schematic overview of the contributing factors and the possible successive effects of ulcerations in the stomach of pigs. Apart from an infection with *H. suis,* the development of gastric ulceration has also been associated with a broad range of factors, such as particle size of the feed, the presence of short fatty acids in the stomach, concurrent diseases and changes in the microbiome. The presence of ulcers in the stomach of pigs may result in decreased feed intake, decreased daily weight gain and fatal hemorrhage. This figure was adapted from Baele et al., 2008 and Haesebrouck et al., 2009.

3.2.2 *H. suis* infections in non-human primates:

Besides in pigs, *H. suis* is also found in the stomach of non-human primates, including cynomolgus monkeys, mandrill monkeys and rhesus macaques (Haesebrouck et al., 2009; Flahou et al., 2016; O'Rourke et al., 2004; Nakamura et al., 2007; Martin et al., 2013; Matsui et al., 2014). This microorganism is mainly localized at the

surface epithelium, the gastric pits and superficial glands of the non-human primate stomach (O'Rourke et al., 2004). Further research is required to determine the exact pathogenic significance of *H. suis* infections in non-human primates (Haesebrouck et al., 2009; Flahou et al., 2016).

3.2.3 *H. suis* infections in experimentally infected laboratory animals

Specific pathogen free (SPF; free of *Helicobacter* spp.) inbred C57BL/6 and BALB/c mice have been shown to be useful models for the study of *Helicobacter*-related gastric disease (O'Rourke and Lee, 2003; Rogers and Fox, 2004). Generally, in experimentally infected mice, inflammation caused by *H. suis* is characterized by the infiltration of mononuclear and polymorphonuclear cells in the lamina propria mucosae and/or the tunica submucosa (**Figure 5A-B**) (Flahou et al., 2010; Bosschem et al., 2016). Infiltration of macrophages can be visualized from 3 weeks post-infection onwards. At 9 weeks post-infection, an augmentation in the number of neutrophils and ($CD3^+$) T-cells can be observed as well (Flahou et al., 2010). At 8 months post-infection large lymphoid aggregates were observed in the stomach of *H. suis*-infected rodents (**Figure 5C**) (Flahou et al., 2010). Apart from inflammation, *H. suis* infections in mice have also been shown to cause necrosis of parietal cells and an increase in mucosal epithelial cell proliferation (Flahou et al., 2010; O'Rourke et al., 2004; Nakamura et al., 2007). Furthermore, gastric lymphoid nodules, hyperplasia of the gastric mucosa and mucus metaplasia, have been described as well after long term (>18 months) infection of mice with *H. suis* (O'Rourke et al., 2004; Park et al., 2008; Matsui et al., 2014). C57BL/6 and BALB/c mice genetically are predominant Th1 and Th2 responders, respectively (Flahou et al., 2010). Differences in gastric inflammation and colonization after *H. suis* infection have been found in these mouse strains. A more pronounced gastric inflammation has been described in the BALB/c mice compared to C57BL/6 mice after infection with *H. suis*, whereas a higher colonization density (both in antrum and fundus) has been noted in *H. suis*-infected C57BL/6 mice compared to *H. suis*-infected BALB/c mice.

Mongolian gerbils are yet another useful rodent model to study *Helicobacter*-related gastric pathology. In these animals, inflammation and colonization is more confined to the antrum and a narrow zone at the forestomach-stomach transition zone, which resembles the antrum predominant colonization by NHPH in humans. In addition, lesions observed in Mongolian gerbils infected with *H. suis* develop faster and are more pronounced than those in mice (Flahou et al., 2010; Bosschem et al., 2016). This includes the relatively fast development of severe inflammation-related pathologies, such as gastric MALT-lymphoma-like lesions. They already appear from 9 weeks post-infection onwards (**Figure 5D**) (Flahou et al., 2010; Bosschem et al., 2016). Humans suffering from a NHPH gastritis have also been shown to develop gastric MALT-lymphoma more frequently than those suffering from *H. pylori* gastritis (Stolte et al., 2002). These findings indicate that the outbred Mongolian gerbil may be more suitable than mice to study interactions of *H. suis* with its human host . Unfortunately, less molecular tools are available for the study of *Helicobacter*-related gastric pathology in gerbils compared to mice.

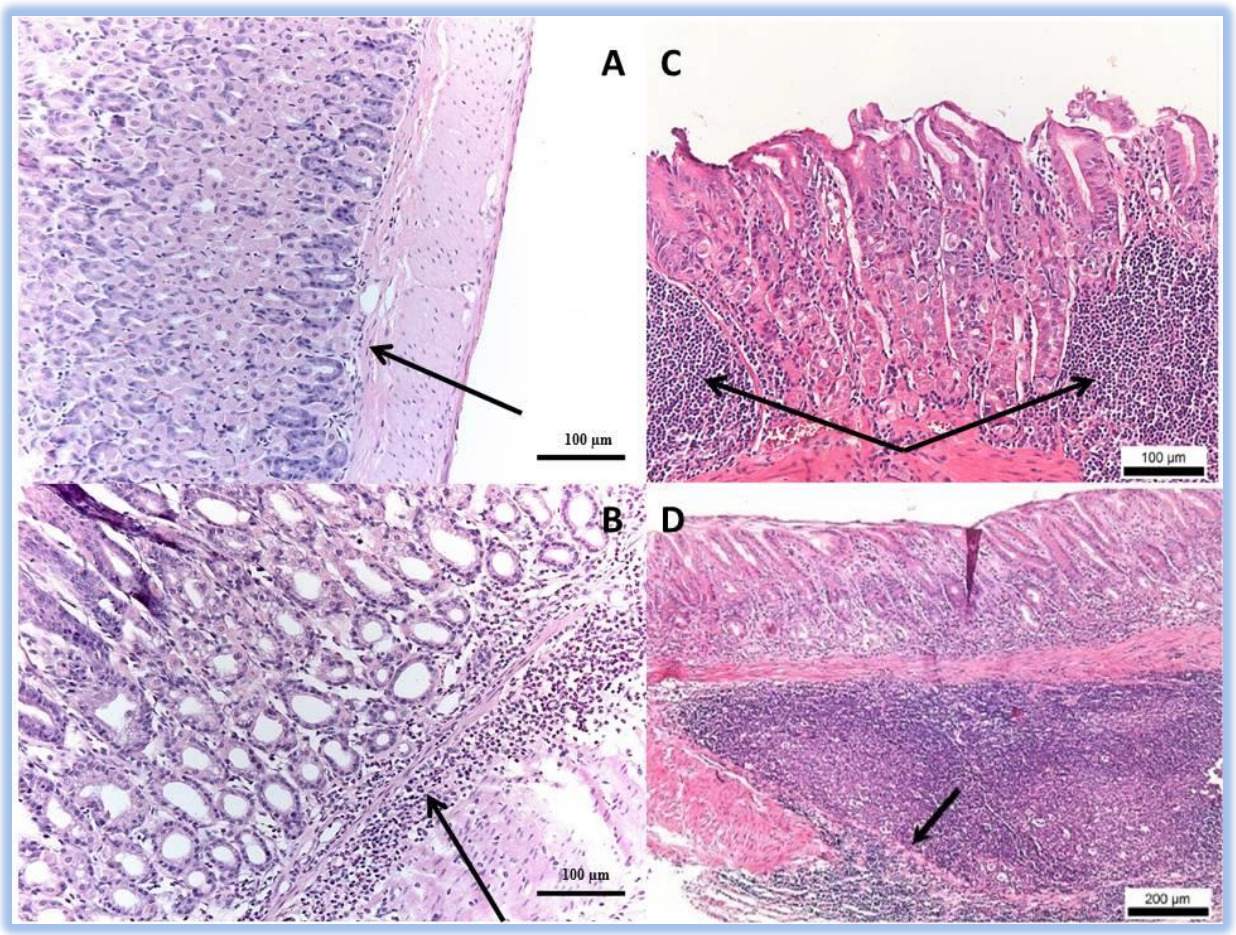

Figure 5: Images of HE stainings of the stomach of mice (A-C) and a Mongolian gerbil (D). **(A)** HE staining of the fundus of C57BL/6 mouse 4 weeks after inoculation of the growth medium of *H. suis* (control mouse). The arrow points to the normal composition and architecture of the mucosa of the fundus, characterized by no or negligible infiltration of mononuclear and polymorphonuclear cells in the lamina propria mucosae and tunica submucosa; **(B)** HE staining of the fundus of C57BL/6 mouse 4 weeks after infection with a porcine *H. suis* strain. The arrow appoints the infiltration of mononuclear and polymorphonuclear cells in the lamina propria mucosae and tunica submucosa; **(C)** HE staining of the fundus of BALB/c mouse 8 month after infection with a porcine *H. suis* strain. The black arrows show the two large lymphoid aggregates in the lamina propria mucosae as a result of major infiltration of mononuclear and polymorphonuclear cells in the lamina propria mucosae in combination with a loss of the normal mucosal architecture. **(D)** HE staining of the antrum of a *H. suis*-infected Mongolian gerbil at 8 months post-infection. The arrow shows the infiltration of mononuclear cells in the tunica muscularis (adapted from Flahou et al., 2010).

3.2.3 *H. suis* infections in man

H. suis is considered to be the most prevalent gastric NHPH species in humans (Haesebrouck et al., 2009). Infections with this zoonotic bacterium have been shown to comprise 13.9-78.5% of all diagnosed human gastric NHPH infections (Trebesius et al., 2001; Van den Bulck et al., 2005; Haesebrouck et al., 2009). It is not exactly known how *H. suis* is transmitted from pigs to humans, but direct and indirect contact with pigs and pork carcasses, as well as the consumption of raw or undercooked pig meat have been considered risk factors (Meining et al., 1998; Van den Bulck et al., 2005; Haesebrouck et al., 2009; Joosten et al., 2013; De Cooman et al., 2013). Similar to other gastric NHPH infections, infections with *H. suis* in humans have been associated with gastric disease and clinical signs may include epigastric pain, nausea, hematemesis, vomiting, irregular defecation frequency and consistency, recurrent dyspepsia, heartburn and decreased appetite (Dieterich et al.,

1998; Goddard et al., 1997; Heilmann and Borchard, 1991; Kaklikkaya et al., 2002; Mention et al., 1999; Oliva et al., 1993; Schildt et al., 2000; Seo et al., 2003; Sykora et al., 2003; Van Loon et al., 2003; Wegmann et al., 1991; Yang et al., 1998; Yoshimura et al., 2002; Dobbs et al., 2005). A recent case report described an *H. suis* infection in a pig veterinarian suffering from reflux esophagitis and general dyspeptic symptoms (Joosten et al., 2013). Diagnosis in this patient was performed using *ureA*-based PCR on gastric biopsy samples and sequencing of the PCR amplicons. Additionally, histological examination revealed the presence of spiral-shaped bacteria in the lumen of the gastric glands as well as the presence of chronic, diffuse gastritis, characterized by a pronounced infiltration of lymphocytes and macrophages (Joosten et al., 2013). Apart from gastritis, *H. suis* infections in humans have also been associated with gastric and duodenal ulcers and MALT-lymphoma (Haesebrouck et al., 2009, Matsumoto et al., 2014). Treatment of human *H. suis* infections generally consists of the administration of amoxicillin, clarithromycin and a proton pump inhibitor (Joosten et al., 2013). However in case of therapy failure, the administration of tetracycline might be recommended (Dobbs et al., 2005).

Due to the extremely difficult diagnosis, the knowledge on human *H. suis* infections is rather limited. Further research is recommended in order to obtain more insights on the pathogenesis and clinical significance of *H. suis* infections in human patients.

3.3 Use of genomics and genetics to obtain better insights in the pathogenesis of *H. suis* infections

Comparison of the recent available whole genome sequences of NHPH, including *H. suis,* with that of *H. pylori* has revealed the presence of genes encoding homologues of *H. pylori* virulence factors involved in acid acclimation, motility, chemotaxis, oxidative stress resistance and gastric epithelial cell damage (Arnold et al., 2011; Schott et al., 2011; Vermoote et al., 2011; Smet et al., 2013; Joosten et al., 2015). An overview is shown in **Table 3**.

Table 3: Overview of NHPH genes encoding homologues of *H. pylori* virulence factors and their role in the pathogenesis of *Helicobacter* infections.

NHPH gene homologues encoding *H. pylori* virulence factors	Proteins	Function
ureA/B/I/E/F/G/H	- UreA and UreB - urease accessory proteins (UreEFGH) - UreI	- acid resistance - regulation of urease activity - acid activated urea channel
che, tlp	- chemotaxis proteins (*che*) - methyl-accepting chemotaxis proteins (*tlp*)	Chemotaxis
katA, sodB, Ahpc	- catalase (KatA) - superoxide dismutase (SodB) - alkyl hydroperoxide reductase (HhpC)	oxidative stress resistance
napA	- neutrophil-activating protein (NAP)	oxidative stress
Ggt	- γ-glutamyl transpeptidase	induction of gastric lesions (apoptosis-inducing and immunosuppressive)
fldA	- flavodoxin protein (FldA)	electron acceptor of the pyruvate oxidoreductase enzyme complex
porA/B/C/D	- pyruvate oxidoreductase complex (Por)	electron donor for FldA
pgbA/B	- plasminogen-binding protein (Pgb)	induction of gastric lesions
ansB	- L-asparginase II	cell-cycle inhibition of fibroblasts and gastric cell lines

3.3.1 pH homeostasis

Like all gastric *Helicobacter* spp., the genome of *H. suis* contains genes encoding the urease enzyme, which consists of two subunits (*UreA* and *UreB*) and plays a role in acid resistance by converting urea into ammonia and carbon dioxide (Eaton et al., 1991; Burn and Chen, 2000). By neutralizing the hydrochloric acid, the ammonia induces an elevated pH in the stomach (Eaton et al., 1991). Additionally, ammonia reduces the viability of the gastric epithelial cells (Smoot et al., 1990). The second urease gene clusters consists of the urease accessory *ureEFGH* genes and the *ureI* gene, encoding for the UreEFGH and the UreI proteins, respectively (Akada et al., 2000). The UreI protein forms an acid-activated urea channel, thereby controlling the transport of urea into the bacterial cell (Scott et al., 2000). In order to be functional, two nickel molecules (Ni^{2+}) should be present within the metallocenter of the *H. pylori* urease (Ge et al., 2013). Together the UreEFGH proteins are responsible for the incorporation of nickel, thereby regulating the urease activity (Cussac et al., 1992; Mobley et al., 1995).

3.3.2 Chemotaxis and motility

Genes coding for proteins involved in the chemotactic behavior (*che* and *tlp* genes) are typical for gastric helicobacters (Arnold et al., 2011; Schott et al., 2011; Vermoote et al., 2011; Smet et al., 2013; Joosten et al., 2015). They allow bacterial movement towards urea, cholesterol, arginine and bicarbonate and away from hydrochloric acid (Foynes et al., 2000; McGee et al., 2005; Terry et al., 2005; Lertsethtakarn et al., 2011). Moreover, the presence of the TlpB receptor, which is required for pH-taxis, is also needed for the colonization of *Helicobacter* spp. (O'Toole et al., 1994; Kim et al., 1999).

All gastric *Helicobacter* spp. are able to migrate to the epithelial cell surface of the stomach and towards a more neutral pH using flagellae (Salama et al., 2013; Arnorld et al., 2011; Schott et al., 2011; Vermoote et al., 2011, Smet et al., 2011). The flagellae are composed of a body and flagellar filaments, consisting of two flagellins A (FlaA) and B (FlaB), a flagellar hook (FlgE) and a flagellar cap (FlgD) (Josenhans et al., 1995; Andrutis et al., 1997; O'Toole et al., 1994; Kim et al., 1999).

Both flagellum dependent motility and chemotaxis are important for successful colonization of the stomach (Moens and Vanderleyden, 1996; Otteman et al 1997).

3.3.3 Oxidative stress

Catalase (*KatA*), superoxide dismutase (*SodB*) and alkyl hydroperoxide reductase (*AhpC*) allow gastric helicobacters to counteract oxidative stress induced by the host immune response (Seyler et al., 2001; Harris et al., 2002; Olczak et al., 2003; Ernst et al., 2005, Schott et al., 2011; Smet et al., 2011; Arnold et al., 2011; Vermoote et al., 2011). The neutrophil-activating protein (NAP) is another virulence factor present in all gastric *Helicobacter* spp., which is involved in oxidative stress resistance (Arnold et al., 2011; Schott et al., 2011; Vermoote et al., 2011; Smet et al., 2013; Joosten et al., 2015). Additionally it induces the production of reactive oxygen radicals (ROS) (Olczak et al., 2003; de Bernard et al., 2010; Evans et al., 1995) and it is responsible for chemotaxis of neutrophils and monocytes, increased adhesion of neutrophils, activation of monocytes, mast cells and neutrophils as well as increasing the lifespan of monocytes and neutrophils (Satin et al., 2000; Montemurro et al., 2001; 2002; Amedei et al., 2006; Polenghi et al., 2007; Cappon et al., 2010).

3.3.4 Outer membrane proteins

Adhesion plays a crucial role in the initial colonization of the stomach by *Helicobacter* (McGuckin et al., 2011). Approximately 64 outer membrane proteins (OMPs) have been identified in the genome of *H. pylori.* Their role in the colonization of the stomach has been studied thoroughly (Alm et al., 2000; Odenbreit et al., 2009). These OMPs can be divided into 5 families. The first family consists of the *Helicobacter* outer membrane porin (Hop) and the Hop related (Hor) proteins. The second is the *Helicobacter* OMP family (Hof) protein family. The third is the *Helicobacter* outer membrane (Hom) protein family. The fourth is the iron-regulated OMPs family. The fifth is the efflux pump OMPs family (Oleastro and Ménard, 2013). Hop proteins include adhesins and porins, which play an important role in the adhesion of *H. pylori* to the gastric mucosa (Alm et al., 2000; Aspholm et al., 2006; Odenbreit, 2005; Gerhard et al., 2001). The two most important adhesins in *H. pylori* are the blood group antigen binding adhesin A (BabA) and the sialic acid binding adhesin A (SabA). BabA binds to human fucosylated Lewis b group antigen or related terminal fucose residues in blood group A, B and O antigens, expressed on Mucin 1 (MUC1) and Mucin 5AC (MUC5AC), whereas SabA binds to sialylated carbohydrate structures upregulated in the inflamed gastric mucosa (Boren et al., 1993; Ilver et al., 1998; Mahdavi et al., 2002).

Although ca. 55-60 OMPs have been identified in the genome of the gastric NHPH (Joosten et al., 2015; Alm et al., 2000), comparative genomics revealed that none of the genes encoding the *H. pylori* adhesins were present

within the NHPH genomes (Joosten et al., 2015). Genes encoding homologs of all members of the *H. pylori* Hof proteins appear to be present in all NHPH genomes, except for *H. mustelae* (Smet et al., 2014). A recent study showed that the *H. heilmannii* HofE and HofF play a role in the adhesion of this bacterium to gastric mucins and epithelial cells (Liu et al., 2016). Further research, however, is recommended in order to determine which other OMPs contribute to the adhesion process.

3.3.5 Virulence factors contributing to the gastric lesions

Major virulence factors of *H. pylori* involved in the induction of gastric lesions are the cytotoxin-associated gene pathogenicity island (*cag*PAI) and the vacuolating cytotoxin A (*VacA*). Both virulence factors are, however, absent in all NHPH with the exception of *H. cetorum* carrying a functional VacA toxin (Vermoote et al., 2011). *Cag*PAI consists of 30 genes encoding a type IV secretion system (T4SS), which penetrates the epithelial cells of the stomach, allowing injection of the CagA protein into the host cells. Phosphorylation of this protein induces morphological changes in the epithelial cells by changes in the cytoskeleton and indirect activation of the pro-inflammatory nuclear factor (NF)-κB (Akopyants et al, 1998; Segal et al., 1999; Asahi et al., 2000; Christie and Vogel, 2000; Odenbreit et al., 2000; Fisher, 2001; Yamazaki et al., 2003; Brandt et al., 2005; Mimuro et al., 2007; Backert et al., 2008). VacA, on the other hand, induces vacuolization of the epithelial cells and mitochondrial-mediated apoptosis. The toxicity of this protein depends on the formation of membrane channels, which are induced by this protein (Cover and Blaser, 1992; Szabo et al., 1999; Kimura et al., 1999, Willhite and Blanke, 2004).

Another virulence factor, which on the contrary is present in all gastric *Helicobacter* species is the gamma-glutamyltranspeptidase (GGT) (Arnold et al., 2011; Vermoote et al., 2011; Schott et al., 2011; Smet et al., 2013). For *H. pylori*, it has been shown that GGT causes damage to epithelial cells by inducing a depletion of glutamine and gluthatione, both important for the maintenance of healthy gastrointestinal tissue (Shibayama et al., 2003; 2007). Additionally, it inhibits T cell proliferation and induces an upregulation of cyclo-oxygenase (COX)-2 and epithelial growth factor (EGF)-related peptide as well as apoptosis of human gastric adenocarcinoma (AGS) cells (Busiello et al., 2004; Schmees et al., 2007; Kim et al., 2007; 2010; Zhang et al., 2015). The contribution of the *H. suis* GGT in apoptotic and necrotic cell death of gastric epithelial cells has been described as well. By the disintegration of glutathione into pro-oxidant degradation products, *H. suis* GGT induces increased oxidative stress, resulting in lipid peroxidation and finally gastric epithelial cell death (Flahou et al., 2011).

Besides VacA, CagPAI and GGT, other virulence factors have been associated with gastric lesions. These include PutA, PutP, AnsB, FldA, PgbA, PgbB, HtrA, PrtC and IceA. Most of them are present in all gastric *Helicobacter* spp. (Arnold et al., 2011; Schott et al., 2011; Vermoote et al., 2011; Kersulyte et al., 2013; Smet et al., 2013; Joosten et al., 2015). These factors have been studied in *H. pylori*. L-Asparginase II (AnsB) plays a role in the cell-cycle inhibition of fibroblasts and gastric cell lines (Scotti et al., 2010). FldA functions as an electron acceptor for the pyruvate oxidoreductase enzyme complex, whereas pyruvate oxidoreductase (Por) proteins function as electron donors for FldA. The presence of the FldA protein has been associated with MALT-lymphoma (Chang et al., 1999). The plasminogen-binding proteins (PgbA and PgbB) will allow *Helicobacter* to

bind to host plasminogen, which may contribute to the obstruction of the natural healing process of gastric ulcers (Vermoote et al., 2011). Furthermore, HtrA is involved in cleavage of E-cadherin, and thus contributes to the disruption of the gastric epithelial barrier (Hoy et al., 2010; Lower et al., 2011). By the production of collagenase (PrtC), gastric helicobacters are able to induce the degradation of collagen (Zhang et al., 2015). Finally, IceA has been associated with peptic ulcer disease and seems to be induced by contact between epithelial cells and *Helicobacter* (Peek et al., 1998; 2000; Donahue et al., 2000).

Although a substantial number of genes encoding putative virulence factors has been described in the NHPH genomes in general and the *H. suis* genome in particular, for the majority of these, their exact role in pathogenesis still remains unclear.

3.4 Host immune response to gastric *Helicobacter* infections

In general, *Helicobacter* causes a life-long gastric infection since the evoked host immune response is not able to eliminate *Helicobacter* from the stomach (Kusters et al., 2006; Flahou et al., 2012). Several studies describing the host immune response (both innate and adaptive host responses) against *H. pylori* are available, whereas only few reports have been published on the host immune responses against *H. suis* infections (Baele et al., 2008; Flahou et al., 2010; Flahou et al., 2012; Bosschem et al., 2016). The next section is therefore particularly focused on the innate and adaptive host immune response induced by *H. pylori* and where possible, information about *H. suis*-induced immune responses is provided as well.

3.4.1 Innate immune response to *H. pylori* and *H. suis*

Dendritic cells (DC), macrophages and gastric epithelial cells form the first barrier against infections with *H. pylori* (Dubois and Borén, 2007). Like other invading pathogens, *H. pylori* bacteria are recognized by the cells of the innate immune system via distinct conserved structures, also referred to as pathogen-associated molecular patterns (PAMPs). These PAMPS are not expressed by the host cells, allowing recognition of pathogens by the host immune response (Takeda et al., 2004; Peek et al., 2010). The host cells, on their account, feature pattern recognition receptors (PRRs) on the cytoplasmatic and endosomal membranes as well as in the cytosol. These PRRs, including the surface-expressed Toll-like receptors (TLRs), are able to recognize the bacterial PAMPs. Specific interactions between PRRs and PAMPs result in the activation of intracellular signaling pathways inducing the transcription of various genes that are responsible for the production of inflammatory cytokines, chemokines, antigen-presenting molecules, etc (Peek et al., 2010). Gastric epithelial cells from *H. pylori*-infected human patients have been shown to express TLR4, TLR5 and TLR9 (**Figure 6A**) (Schmausser et al., 2004). TLR2 is generally expressed on the surface of intestinal and gastric epithelial cells (Cario et al., 2002; Smith et al., 2003) and in contrast to TLR4, TLR5 and TLR9, it is responsible for the recognition of multiple *H. pylori* PAMPs, *e.g.* lipopolysaccharide (LPS) and the *H. pylori* neutrophil-activating protein (HP-NAP) (Peek et al., 2010) (**Figure 6A**). Interaction of HP-NAP with TLR2 leads to the activation of NF-κB, followed by the upregulation of pro-inflammatory cytokines interleukin (IL)-12, IL-23 and tumor necrosis factor (TNF) (Amedei et al., 2006). (**Figure 6A**).

Since *H. pylori* possesses 4 to 6 monopolar sheated flagella and TLR5 is known to recognize bacterial flagellin, TLR5 was assumed to be involved in the recognition of *H. pylori* flagellin (Eaton et al., 1996). However, full TLR5 activation requires the presence of conserved amino acids. Because of the lack of these molecules, it has been reported that *H. pylori* flagellin cannot be recognized by TLR5 (Andersen-Nissen et al., 2005) (**Figure 6A**).

Additionally to TLRs, gastric epithelial cells may express the cytosolic nucleotide-binding oligomerization domain (NOD), which is a cytosolic pathogen recognition molecule. By its involvement in the intracellular recognition of bacterial muropeptides, derived from bacterial peptidoglycan, NOD1 plays an important role in the innate immunity against *H. pylori*. Using the type 4 secretion system (T4SS), *H. pylori* secretes its peptidoglycan into the cytoplasma of the gastric epithelial cells, resulting in the activation of NOD1 (Viala et al., 2004). *H. pylori*-induced NOD1 activation results in the activation of NF-κB, leading to the production of pro-inflammatory cytokines and chemokines, eg CXCL-8 (IL-8), CXCL-2 (murine homologue of IL-8) and type 1 interferon (IFN) (Viala et al., 2004; Watanabe et al., 2011) (**Figure 6A**).

Furthermore, IL-8 production by gastric epithelial cells can also be mediated in a TLR/NOD1 independent manner by interaction with *H. pylori* OMPs (Oleastro & Menard, 2013) (**Figure 6A**). Via BabA and SabA adhesins*, H. pylori* is also able to bind to gastric mucins, such as MUC1, which also results in the secretion of IL-8 by the gastric epithelial cells (**Figure 6A**) (Sheng et al., 2013). Additionally, interaction between the gastric epithelial cells and other *H. pylori* adhesins, including HopH, outer inflammatory protein A (OipA), HomB and adherence-associated lipoprotein A/B (AlpA/AlpB), may also induce gastric epithelial IL-8 secretion (Oleastro &Menard, 2013; Sheng et al., 2013) (**Figure 6A**).

The production of chemokines by the epithelial cells of the stomach results in the recruitment of innate immune cells, eg. dendritic cells, monocytes, macrophages and neutrophils to the site of infection. They are considered to be the second stage of the innate immune response (Allen et al., 2000; 2001). Interaction between *H. pylori* and monocytes/macrophages results in the activation of both TLR2 and TLR4 signaling in these cells, leading to the secretion of inflammatory cytokines such as IL-1β, IL-6, IL-8, IL-10, IL-12 and TNF (Maeda et al., 2001; Gobert et al., 2004; Mandell et al., 2004). On the other hand, DC signaling in response to an *H. pylori* infection is mainly mediated by TLR2 and to a lesser extent by TLR9 and TLR4, inducing the secretion of IL-1β, IL-6, IL-10, IL-12 and TNF (Rad et al., 2007; 2009; Kim et al., 2013). The production of these pro-inflammatory cytokines by DC's and macrophages leads to the recruitment, differentiation and activation of $CD4^+$ Th cells, which organize the adaptive immune response (Wilson & Crabtree, 2007). Furthermore, macrophages and neutrophils are responsible for phagocytosis, which is an important antibacterial innate defense mechanism. However, by its ability to survive within the phagosomes, *H. pylori* partially evades phagocyte-mediated killing (Allen et al., 2000; 2001). In addition, *H. pylori* induces the extracellular release of ROS by phagocytes, but the bacterium is capable of surviving this response through catalase and superoxide dismutase activity (Ramarao et al., 2000).

Little data is available on the innate immune response against an infection with *H. suis.* A recent *in vivo* study reported infiltration of neutrophils and mononuclear cells in the mucosa and submucosa of *H. suis*-infected BALB/c mice (Bosschem et al., 2016) (**Figure 6B**). The same study described enhanced expression of porcine DC

activation markers (CD25, CD40 and CD80/86), decreased expression of major histocompatibility complex (MHC)-II presentation at the surface of porcine DCs and DC-triggered expansion of regulatory T cells (Tregs) upon stimulation with *H. suis* lysate. Finally, *H. suis* stimulation of murine DCs resulted in an increased secretion of IL-6 and IL-23, inducing the differentiation from naïve T cells to T helper 17 (Th17) effector cells (Bosschem et al., 2016) (**Figure 6B**). Since no information is currently available on *H. suis* PAMPs (**Figure 6B**), further research is required to identify the *H. suis* genes that contribute in the activation of the host immune response.

3.4.2 Acquired immune response to *H. pylori* and *H. suis*

In response to the cytokine production by the innate immune cells, naïve T $CD4^+$ helper (T $CD4^+$) cells will differentiate towards either T helper 1 (Th1) cells, T helper 2 (Th2) cells, Th17 or regulatory T (Treg) cells. An overview of the cytokines responsible for the differentiation of naïve T $CD4^+$ cells into the different effector cells in combination with the secretion of cytokines by the different T effector cells is presented in **Table 4**.

Table 4: Overview of the cytokines responsible for the differentiation of naïve T $CD4^+$ cells into the different T effector cells and their secreted cytokines.

	Th1 effector cells	Th2 effector cells	Th17 effector cells	Treg effector cells
cytokines for differentiation	- IL-12	- IL-6/ IL-4	- IL-6/ IL-23	- TGF-β
secreted cytokines	- IFN-γ/ IL-2/ TNF	- IL-4/ IL-5/ IL-6/ IL-9/ IL-10/ IL-13	- IL-17/ IL-21/ IL-22	- IL-10/ TGF-β
references	Romagnani et al., 2000; Larussa et al., 2015 ; Banchereau et al., 2000	Romagnani et al., 2000; Larussa et al., 2015 ; Banchereau et al., 2000	Larussa et al., 2015	Larussa et al., 2015

3.4.2.1 T helper cells

The T cell response evoked by *H. pylori* infections consists of the IFN-γ producing $CD4^+$ T cell phenotype, indicating a Th1 response (D'Elios et al., 1997; Bamford et al., 1998; Peek et al., 2010; Larussa et al., 2015). Differentiation of the naïve T $CD4^+$ helper cells into Th1 helper cells is induced by IL-12 (**Table 4**), produced and secreted by activated monocytes, macrophages and DCs (Guiney et al., 2003; Hafsi et al., 2004) (**Figure 6A**). The severity of *H. pylori*-associated gastric pathology mainly depends on the Th1 responses and the Th1 produced cytokines (Mohammadi et al., 1996; Smythies et al., 2000; Sommer et al., 2001). These findings have been confirmed by the reduced gastric pathology in mouse models deficient in Th1 cell development (Sommer et al., 2001) and IFN-$\gamma^{-/-}$ mouse models (Smythies et al., 2000). Several *H. pylori* virulence factors have been associated with the stimulation of the Th1 cell response, including CagPAI and HP-NAP. *H. pylori* infections have also been associated with a Th2 response (Serrano et al., 2007; Robinson and Atherton, 2010), characterized by the secretion of multiple cytokines including IL4, IL6 and IL10 (**Table 4**)(Kusters et al., 2006; Smythies et al., 2000; Odenbreit et al., 2006). *H. pylori* strains inducing a Th2 immune response typically lack genes encoding

major virulence factors such as the CagPAI and the VacA and the BabA and SabA adhesins (Liu et al., 2014). Apart from the production of multiple cytokines (**Table 4**), activated Th2 cells are also responsible for the inhibition of phagocytic cells (Romagnani et al., 2000). Indeed, several reports suggest the protective role of Th2 cells against severe lesions by suppressing the *H. pylori*-induced inflammation (Fox et al, 2000; Smythies et al., 2000). Other reports could not confirm these findings (Saldinger et al., 1998; Garhart et al., 2003) and further research is required to elucidate the exact role of the Th2 response in *H. pylori* infections.

A Th2 host immune response has also been associated with an *H. suis* infection in mice (Park et al., 2008). This type of response was characterized by elevated expression levels of IL-10 and IL-4 (Flahou et al., 2012; Zhang et al., 2015) (**Figure 6b**), as well as by the formation of gastric MALT-lymphoma-like lesions (Greiner et al., 1997; Flahou et al., 2012). Gastric MALT-lymphoma is characterized by a strong proliferation of B lymphocytes which may be dependent on Th2-type cytokines. Since infection with NHPH in general and *H. suis* in particular results indeed in a higher risk for the development of MALT-lymphoma, it is more likely to induce a Th2 response rather than a Th1 response (Greiner et al., 1997; Knörr et al., 1999; Haesebrouck et al., 2009; Flahou et al., 2010; Flahou et al., 2012). On the other hand, infections with *H. suis* in gerbils result in increased expression of IFN-γ in the stomach, suggesting the induction of a Th1 response by this pathogen as well (Vermoote et al., 2012; 2013; Bosschem et al., 2015; Zhang et al., 2015) (**Figure 6B**). These results indicate that the *H. suis*-evoked immune response might be host dependent. However, further investigation is recommended.

Another important immune mechanism contributing to the development of *H. pylori*-induced gastritis is the Th17 response (Shiomi et al., 2008; Shi et al., 2010; Hitzler et al., 2012; Raghavan and Quiding-Järbrink, 2012; Gray et al., 2013; Larussa et al., 2015). Th17 cells secrete IL-17, which plays a role in the recruitment of neutrophils to the site of infection, as well as in the stimulation of IL-8 secretion by the gastric epithelial cells (Luzza et al., 2000; Mizuno et al., 2005) (**Figure 6A**).

An infection with *H. suis* has also been associated with a Th17 response. The presence of Th17 cells and/or increased levels of IL-17 in the stomach of *H. suis*-infected mice, Mongolian gerbils and humans has been reported in several studies (Luzza et al., 2000; Mizuno et al., 2005, Flahou et al., 2012; Vermoote et al., 2012; 2013; Zhang et al., 2015; Bosschem et al., 2015; 2016) (**Figure 6B**). However, the Th17 response upon *H. suis*-infected mice appeared to be significantly higher in BALB/c mice than in C57BL/6 mice (Flahou et al., 2012). This finding can be explained by the fact that the overall inflammatory response in BALB/c mice is generally higher compared to C57BL/6 mice (Flahou et al., 2012). Additionally, a remarkable negative correlation between *H. suis* colonization and the expression levels of IL-17 mRNA has been reported (Flahou et al., 2012; Vermoote et al., 2012; 2013; Bosschem et al., 2015). Indeed, the higher inflammatory response in BALB/c mice might induce a less favorable environment for *H. suis,* thereby resulting in a lower colonization density (Flahou et al., 2012).

An increased number of Treg cells in the gastric mucosa of *H. pylori*-infected human patients has been described, suggesting that *H. pylori* colonization could evoke proliferation and migration of Treg cells as well (Lundgren et al., 2005). FOXP3, a gene contributing to the development of Tregs, is expressed by the $CD4^+$ $CD25^+$T cells in the gastric and duodenal mucosa of *H. pylori*-infected patients (Lundgren et al., 2005) (**Figure**

6A). By the production of anti-inflammatory cytokines (IL-10 and TGF-β), Treg cells are able to limit the proliferation and function of other immune cells, which may result in decreased inflammation and tissue damage (Eaton et al., 2001; Raghavan and Quiding-Järbrink, 2012). Indeed, Treg responses in *H. pylori*-infected children resulted in the inhibition of the Th1 and Th17 responses leading to a less severe inflammation and ulceration of the gastric mucosa (Harris et al., 2008). Moreover, the number of Treg cells was considerably higher in *H. pylori*-infected patients without peptic ulceration compared to patients suffering from peptic ulcers (Robinson et al., 2008).

H. suis infections in mice and gerbils have also been associated with the induction of a Treg immune response and increased expression levels of IL-10 in the gastric mucosa (Vermoote et al., 2012; 2013; Zhang et al., 2015; Bosschem et al., 2015; 2016). Elevated expression of IL-10 and the FOXP3-gene were observed in *H. suis*-infected mice (Zhang et al., 2015) (**Figure 6B**). Additionally, a positive correlation between IL-10 expression levels and *H. suis* colonization density has been described in BALB/c mice (Vermoote et al., 2012; 2013; Flahou et al., 2012).

3.4.2.2 B-cells

It has been suggested that B cells may also contribute to the immunopathogenesis of *H. pylori* infections. Uncontrolled proliferation of B lymphocytes has been reported in response to the production of certain cytokines, like IL-2, after interaction between T cells and *H. pylori* antigens (Wotherspoon et al., 1991). A minority of *H. pylori*-infected patients develop gastric MALT-lymphoma, which mainly arise by the proliferation of B cells (Kusters et al., 2006). Moreover, complete MALT-lymphoma regression has been described in more than 80% of the patients after successful *H. pylori* eradication (Parsonnet and Isaacson, 2004; Peek et al., 2010). Compared to wild-type mice, a reduced colonization and more severe gastritis combined with the increased infiltration of $CD4^+$ cells was detected in B cell deficient mice at 8 and 16 weeks post-infection, indicating that B cells may temper the effect of the $CD4^+$ cells (Akhiani et al., 2004). Further research is recommended to uncover the exact role of B cells as well as the role of T cell-B cell interactions in the pathology of the *H. pylori*-evoked immune response (Peek et al., 2010).

Although the exact role of B cells in *H. suis* infections remains to be elucidated, previous studies suggested that they might play a role in the pathogenesis as well (Vermoote et al., 2012; 2013, Zhang et al., 2015) (**Figure 6B**). Increased numbers of B cells have been reported after a 6 month-infection of mice and Mongolian gerbils with *H. pylori* and *H. suis* (Zhang et al., 2015). Inoculation of C57BL/6 mice with an *H. suis* strain evoked a host immune response characterized by activation and infiltration of B cells and $CD4^+$ cells, which were mainly located in the surrounding of gastric lymphoid follicles. Moreover, their numbers were positively correlated with the duration of *H. suis* infection (Yamamoto et al., 2011). Additionally, a pronounced upregulation of chemokine ligand 13 (CXCL13), also called B cell attracting chemokine 1 or B lymphocyte chemoattractant, along with infiltration and proliferation of B cells in the centers of lymphocytic aggregates/follicles has been reported in the stomach of *H. suis*-infected, but not *H. pylori*-infected mice and Mongolian gerbils (Zhang et al., 2015) (**Figure 6B**). In contrast, another study showed that *H. suis*-induced lymphoid follicles were not only

composed of B cells but also of $CD4^+$ T helper cells and that the development of gastric lymphoid follicles could not be detected in *H. suis*-infected IFN-γ deficient mice, indicating that IFN-γ might play a role in the pathogenesis of *H. suis*-induced gastric lymphoid follicles (Mimura et al., 2011).

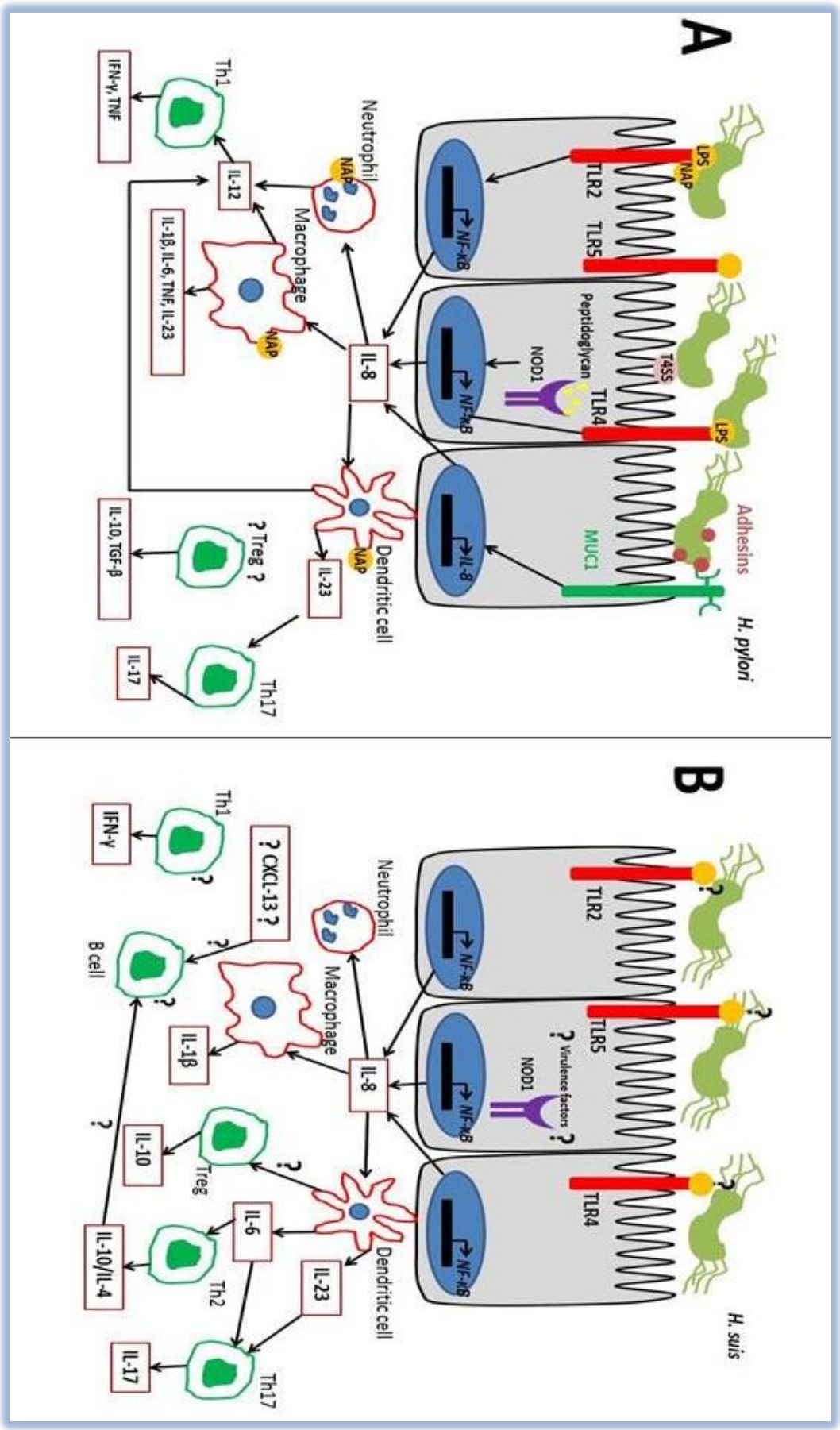
A
LPS
NAP
TLR2
TLR5
NF-κB
Peptidoglycan
NOD1
T4SS
TLR4
LPS
IL-8
MUC1
Adhesins
H. pylori
Neutrophil
NAP
Macrophage
NAP
IL-8
Dendritic cell
NAP
IL-23
Th1
IL-12
IFN-γ, TNF
IL-1β, IL-6, TNF, IL-23
? Treg ?
IL-10, TGF-β
Th17
IL-17
B
TLR2
TLR5
? Virulence factors ?
NOD1
TLR4
NF-κB
H. suis
Neutrophil
Macrophage
IL-8
Dendritic cell
? CXCL-13 ?
Th1
IFN-γ
B cell
IL-1β
IL-10
Treg
IL-6
IL-23
IL-10/IL-4
Th2
Th17
IL-17

Figure 6: Comparison of *H. pylori*-induced and *H.* suis-induced innate and adaptive immune response. (**A**) Overview of the *H. pylori*-evoked host innate and adaptive immune response. Contact between epithelial cells and *H. pylori* or its bacterial products may lead to the activation of NF-κB, which results in the secretion of pro-inflammatory chemokines and cytokines, including IL-8. IL-8 induces the recruitment of immune cells to the lamina propria of the gastric mucosa. Depending on their effector functions, activated immune cells secrete pro-inflammatory cytokines (including IL-1β, TNF, IL-6, IL-12, and IFN-γ). Based on these cytokines, naïve T $CD4^+$ cells will differentiate towards either Th1 cells, Th2 cells, Th17 or Treg cells. Depending on the type of T-helper cell, certain cytokines will be secreted. (**B**) Overview of the *H. suis*-evoked host innate and adaptive immune response. Contact between epithelial cells and *H. suis* or its bacterial products may lead to the activation of NF-κB, which results in the secretion of pro-inflammatory chemokines and cytokines, including IL-8 and IL-1β. In contrast to *H. pylori,* the virulence factors of *H. suis* that evoke the innate immune response remain unknown. Subsequently, the pro-inflammatory cytokines induce the recruitment of immune cells to the lamina propria of the gastric mucosa. Depending on their effector functions, activated immune cells secrete pro-inflammatory cytokines, such as IL-23 and IL-6. Based on these cytokines, naïve T $CD4^+$ cells will differentiate towards either Th1 cells, Th2 cells, Th17 or Treg cells. Depending on the type of T-helper cell, certain cytokines will be secreted. In conclusion, only limited information on the *H. suis*–induced innate and adaptive host immune response is currently available and thus further research is required. IL: interleukin; TNF: tumor necrosis factor; TLR: Toll-like receptor; IFN: interferon; Th: T-helper cell; NOD: nucleotide-binding oligomerization domain; NF: nuclear factor; CXCL-13: chemokine ligand 13; MUC1: mucin 1; T4SS: Type 4 secretion system; LPS: lipopolysaccharide; TGF: Tissue growth factor; NAP: Neutrophil-activating protein.

4. Extragastric manifestations of *Helicobacter* infections with emphasis on Parkinson's disease (PD)

The bidirectional communication between the gastrointestinal tract and the brain via the gut-brain axis has been considered (Cryan et al., 2012; Mayer et al., 2014). Anatomically, the gut-brain axis consists of the central nervous system (CNS), the autonomic nervous system (ANS) and the peripheral nervous system (PNS) (also known as the enteric nervous system or "little brain") (Caso et al., 2008; Konturek et al., 2011; Budzynski et al., 2014). Via the gut-brain axis, changes in stress and emotions are able to influence the gastrointestinal immune system, the mucosal inflammation and the composition of gut microbiota and thereby the functionality of the gastrointestinal tract (Budzynski et al., 2014). On the other hand, alterations in the gut microbiota and the function and/or coordination of the gastrointestinal tract have been suggested to play a role in the overall physical and emotional state of the host (Cryan et al., 2012; Budzynski et al., 2014; Mayer et al., 2014).

Infections with *H. pylori* have not only been associated with changes in the composition of the gastrointestinal microbiota (Engstrand et al., 2013; Budzynski et al., 2014), but also with disturbed gastric emptying (Miyaji et al., 1999). Additionally, *Helicobacter* spp. in general and *H. suis* in particular are able to cause damage to the gastric epithelial cell lining by inducing epithelial cell apoptosis as described above (De Bock et al., 2006; Kusters et al., 2006; Flahou et al., 2010). Furthermore, *Helicobacter* antigens were also detected in macrophages in the mesenteric lymph nodes during chronic infection in mice (unpublished results). Together, these findings suggest that the inflammatory effects caused by gastric helicobacters are not restricted to the stomach. At the start of this PhD, only one clinical association had been made between *H. suis* infections and extragastric disease (Dobbs et al., 2005). In contrast, *H. pylori* infections had been associated with several

extragastric manifestations. A concise overview is shown in **Table 5**. In this part of the thesis, only the possible link between *H. pylori* infections and Parkinson's disease (PD) will be described in more detail.

Table 5: Concise overview of *H. pylori*-associated extragastric disorders. (MS): multiple sclerosis; (AD): Alzheimer's disease; (CNS): central nervous system; (PD): Parkinson's disease; (IBD): inflammatory bowel disease.

Group	Disease	Concise disease description	Association with *H. pylori*	References
neurological manifestations	ischemic stroke	stroke mainly caused by occlusion of the carotic or cerebral vessels	positive	Yang et al., 2011; Alvarez-Arellano et al., 2014 ; De Bastiani et al., 2008; Zhang et al., 2008; Wang et al., 2012; Wong et al., 2014
	migraine headaches	severe headaches	positive	Deleu et al., 1998; Gervil et al., 1999; Gasbarrini et al., 1998; Yiannopoulou et al., 2007; Tunca et al., 2004 ; Hong et al., 2007
	Guillain-Barré syndrome	acute, inflammatory autoimmune neuropathy characterized by progressive weakening of the motoric functions, mainly commencing in the legs	positive	Suzuki et al., 2006; Alvarez-Arellano et al., 2014 ; Kountouras et al., 2005; Chiba et al., 2002
	multiple sclerosis (MS)	most prevalent inflammatory demyelinating disorder of the central nervous system (CNS)	negative	Wender, 2003; Li et al., 2007; Mohebi et al., 2013
	neuromyelitis optica	disease characterized by inflammation of the optic nerves and the spinal cord	positive	Li et al., 2009; Long et al., 2013
	Alzheimer's disease (AD)	neurodegenerative disorder characterized by synaptic loss and neuronal cell death as a result of the intra- and extracellular accumulations of β-amyloid deposits in the brain	positive	Selkoe, 2002; Kountouras et al., 2006; Honjo et al., 2009; Chang et al., 2013; Alvarez-Arellano et al., 2014; Testerman et al., 2014; Wong et al., 2014; Malaguarnera et al., 2004; Kountouras et al., 2009a; Roubaud-Baudron et al., 2012 ; Kountouras et al., 2010
	Parkinson's disease (PD)	neurodegenerative disorder characterized by progressive loss of domaminergic neurons, mainly in the substantia nigra of the brain	positive	Altschuler, 1996; Tsolaki et al., 2015; Bjarnason et al., 2005; Dobbs et al., 2010; Alvarez-Arellano et al., 2014 ; for review see Dobbs et al., 2016
endocrine manifestations	diabetes	endocrine disorder characterized by a disturbance in the glucose metabolisation	positive	Simon et al., 1989, Oldenburg et al., 1996; Quadri et al., 2000; Gulcelik et al., 2005; Bener et al., 2007; Longo-Mbenza et al., 2007; Devrajani et al., 2010; Zhou et al., 2013; Jeon et al., 2012
	dyslipidemia	manifestation characterized by disturbances in the production and clearance of plasma lipoproteins	positive	Laurila et al., 1999; Hoffmeister et al., 2001; Majka et al., 2002; Takashima et al., 2002; Chimienti et al., 2003; Papamichael et al., 2009; He et al., 2014; Feingold et al., 1992; Chimienti et al., 2003
	autoimmune thyroid disorders	autoimmune mediated impairment of the thyroid gland	positive	Papamichael et al., 2009; Smyk et al., 2014; Testerman et an 2014; Bassi et al., 2010; Bassi et al., 2012; Shi et al., 2013; Tomb et al., 1997; Bassi et al., 2010; Bertalot et al., 2004

hematological manifestations	iron deficiency anemia	anemia as a result of a lack of iron often caused by gastrointestinal bleeding.	positive	Monzon et al., 2013; Blecker et al., 1991; Bruel et al., 1993; Dufour et al., 1993 ; Milman et al., 1998; Peach et al., 1998; Collett et al., 1999 ; Yip et al., 1997; Kang et al., 2011; Musumba et al., 2012
	idiopathic thrombocytopenic purpura	autoimmune hematological disease characterized by low platelet counts and mucocutaneous bleedings caused by the autoimmune destruction of platelets	positive	Gasbarrini et al., 1998; Hino et al., 2003; Huber et a., 2003; Stasi et al., 2005; Suzuki et al., 2005; Campuzano-Maya, 2014 ; Franceschi et al., 2004; Takahashi et al., 2004
cardiopulmonary manisfestations	cardiovascular manifestations, such as atherosclerosis and coronary artery disease	narrowing of the lumen of the blood vessels	positive	Mendall et al., 1994; Huang et al., 2011; Park et al., 2011; Vafaeimanesh et al., 2014; Kowalski et al., 2001; 2002; Oshima et al., 2005; Tamer et al., 2009; Chimienti et al., 2003; Kucukazman et al., 2009; Franceschi et al., 2002
	asthma and allergies	hypersensitivity reaction associated with a chronic Th2 response	negative	Amedei et al., 2010; Testerman et al., 2014; Wong et al., 2014; Chen et al., 2008; Zhou et al., 2013; Wang et al., 2013; Strachan, 1989; 2000
hepatic and biliary manifestations	non-alcoholic fatty liver disease	a group of liver disorders ranging from non-alcoholic fatty liver to the development of non-alcoholic steatohepatitis in which insulin resistance plays a role	positive	Rabelo-Gonçalves et al., 2015; Waluga et al., 2015; Cindoruk et al., 2008; Pirouz et al., 2009; Dogan et al., 2013; Abenavoli et al., 2013
	hepatic fibrosis, cirrhosis	disease characterized by replacement of liver cells by extracellular matrix proteins, including collagen, as a result of chronic liver diseases	positive	Rabelo-Gonçalves et al., 2015; Waluga et al., 2015; Goo et al., 2009; Ki et al., 2010; Ponzetto et al., 2000a; b; Feng et al., 2014; Esmat et al., 2012; Stalke et al., 2001
	hepatic encephalopathy	complication of liver cirrhosis characterized by the occurrence of neuropsychiatric symptoms and the excessive ammonia levels	positive	Amodio et al., 2004; Schulz et al., 2014
	hepatocellular adenocarcinoma	primary malignant liver tumor	positive	Rabelo-Gonçalves et al., 2015; Waluga et al., 2015; Avenaud et al., 2000; Nilsson et al., 2001; Dore et al., 2002; Verhoef et al., 2003; Pellicano et al., 2004; Huang et al., 2004
intestinal manifestations	inflammatory bowel disease (IBD)	IBD, an intestinal disorder of unknown pathogenesis, is considered a multifactorial manifestation mainly resulting from a complex interaction between environmental factors and gut microbiota in genetically susceptible humans	negative	Buchner et al., 2011; Papamichael et al., 2014; Ruuska et al., 1994; Thia et al., 2008; Luther et al., 2010; Rokkas et al., 2015; Tursi, 2006; Guiney et al., 2003; Chen et al., 2008
dermatologic manifestations	chronic spontaneous urticaria	dermatological manifestation of unknown etiology characterized by the spontaneous occurrence of itchy wheals lasting for at least 6 weeks	positive	Zuberbier et al., 2009; Magen et al., 2014; Smyk et al., 2014; Testerman et al., 2014, Wong et al., 2014; Federman et al., 2003; Fukuda et al., 2004; Magen et al., 2007; Akashi et al., 2011; Chiu et al., 2013
	rosasea	chronic skin disorder characterized by persistent	positive	Rebora et al., 1994; Gürer et al., 2002; Powell, 2005 ; Argenziano et al., 2003;

		central facial erythema		Bakar et al., 2007; Mashimo et al., 2006
	psoriasis	autoimmune skin disorder characterized by increased proliferation and less differentiation of the keratinocytes in the epidermis	positive	Magen et al., 2014; Smyk et al., 2014; Testerman et al., 2014; Qayoom et al., 2003; Ali et al., 2008; Martin Hübner et al., 2008, Onsun et al., 2012
	Behçet's disease	autoimmune multisystemic inflammatory vascular disorder featured by uveitis, skin lesions, genital ulcers and recurrent oral aphthous ulcers	positive	Magen et al., 2014; Avci et al., 1999; Pay et al., 2007
ocular manifestations	glaucoma	progressive ocular neuropathy is caused by apoptosis of retinal ganglion cells, leading to modifications of the optical nerve and vision loss	positive	Kountouras et al., 2001; 2002; 2003; Zavos et al., 2012; Tsolaki et al., 2015; Ritch, 2007; Sacca et al., 2014; Davies et al., 1994
	central serous chorioretinopathy	this visual impairment is caused by the leakage of fluid in the subretinal space as a result of the break-down of blood-retina barrier consisting of retinal pigment epithelium	positive	Wang et al., 2008; Casella et al., 2012; Giusti, 2004; Cotticelli et al., 2006; Casella et al., 2012; Dang et al., 2013; Rahbani-Nobar et al., 2011 ; Sacca et al., 2014
autoimmune systemic rheumatic manifestations	Sjögren syndrome	chronic inflammatory autoimmune disorder characterized by lymphoid cell infiltration and destruction of endocrine glands, specifically lacrimal and salivary glands	positive	Hasni et al., 2011; Radic et al., 2014; Aragona et al., 1999; Showji et al., 1996
	rheumatoid arthritis	autoimmune inflammatory disorder with symmetric destructive polyarthritis	negative	Hasni et al., 2011; Radic et al., 2014; Showji et al., 1996; Tanaka et al., 2005; Meron et al., 2010; Janssens et al., 1992
	systemic lupus erythematosus	autoimmune multisystemic inflammatory disorder affecting multiple organ systems such as skin, joints, kidneys, brain, etc	negative	Showji et al., 1996; Sawalha et al., 2004; Hasni et al. 2011, Radic et al., 2014; Yamanishi et al., 2006

4.1 PD: clinical outcome

4.1.1. History and pathological findings

PD was first described in 1817 by James Parkinson as the "shaking palsy", a disorder characterized by tremor, muscle rigidity and poverty and slowness of movement (hypo- and bradykinesia) (Parkinson, 1817) and is the second most common neurodegenerative disorder in the current human population (Wirdefeldt et al., 2011; Mhyre et al., 2012). This neurological manifestation is characterized by a degeneration of the dopamine producing neurons in the pars compacta of the substantia nigra (in the basal ganglia of the brain) (**Figure 7**), thereby inducing dystrophic striatial projections (Wirdefeldt et al., 2011; Mhyre et al., 2012; Kannarkat et al., 2013). Inflammatory processes, characterized by oxidative stress, accumulation of cytokines and activation of microglia cells, have been suggested to contribute to the degeneration of the nigrostriatal pathway and

accelerate the disease progression, especially in case of idiopathic PD, a syndrome of unknown etiology (Tansey et al., 2007; Dobbs et al., 2008). Indeed, neuro-inflammation was more pronounced in the pons, striatum, substantia nigra, and frontal and temporal cortical regions in patients with early PD compared to age-matched control patients (Gerhard et al., 2006), and chronic use of non-steroidal anti-inflammatory drugs (NSAID) has been suggested to lower the risk of PD development (Tansey et al., 2007). Additionally, PD is considered as a chronic neuro-inflammatory disorder characterized by the occurrence of Lewy bodies, which are mainly localized in the surviving neurons. Lewy bodies are formed by the accumulation of intracellular protein aggregates, comprised mainly of misfolded α-synuclein. They are identified as eosinophilic inclusion bodies in neurons (Gibb et al.,1988; McNaught et al., 2003; Wirdefeldt et al., 2011; Mhyre et al., 2012; Kannarkat et al., 2013). Furthermore, an abnormal mitochondrial morphology, characterized by a long, thin appearance, has been observed within the enterocytes of the duodenum of PD patients, whereas impaired functionality has been reported in nigral and platelet mitochondria (Dobbs et al., 2008; Dobbs et al., 2016). Mitochondria are responsible for the production of ROS as a byproduct of molecular oxygen consumption in the electron transport chain. Additionally, these subcellular organelles are targets for cytokines, such as TNF, resulting in the overproduction of ROS by the mitochondria. Finally, cell death may occur as a result of oxidative stress, caused by the intracellular accumulation of ROS (Tansey et al., 2007).

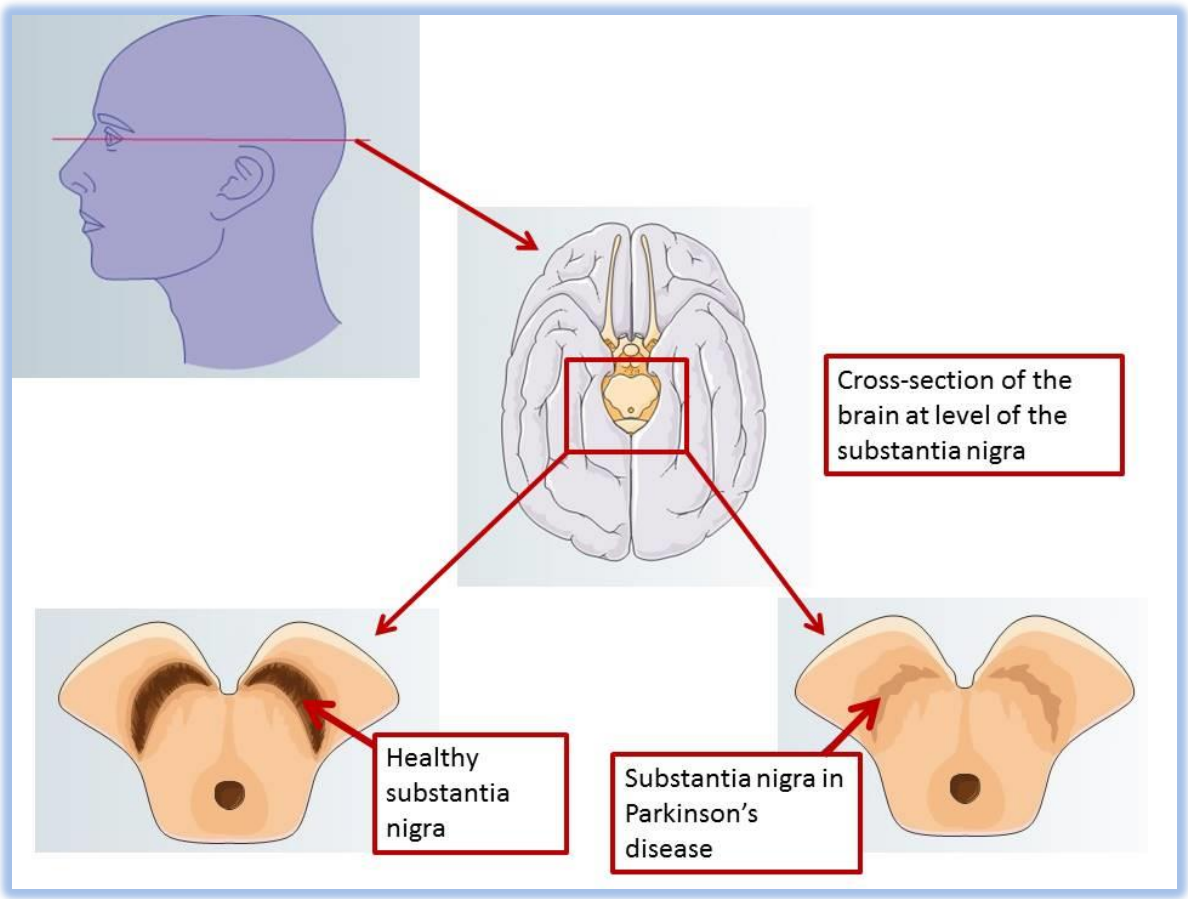

Figure 7: Comparison between a healthy substantia nigra and a diminished substantia nigra in PD, characterized by a loss of dopamine producing neurons.

4.1.2 Motor and non-motor symptoms

This progressive neurodegenerative disorder leads to a broad spectrum of motor and non-motor symptoms. In general, PD-associated motor symptoms, such as resting tremor, muscle rigidity, brady-and hypokinesia and stooped posture are commonly known (Parkinson, 1817; Wirdefelt et al., 2011, Kannarkat et al., 2013). However, degeneration of the majority of the dopamine producing neurons (up to 80%) in the substantia nigra is a prerequisite for the development of motoric symptoms in PD patients (Kordower et al., 2013). In the majority of the patients (60-70%), resting tremor is the first indicative symptom for PD that appears. Generally the tremor has an unilateral and distal character (*e.g.* hand, fingers, foot, forearm), but over time it tends to expand to the contralateral upper limb or ipsilateral lower limb (Wirdefelt et al., 2011; Mhyre et al., 2012). Rigidity has a rather unilateral character and is generally more pronounced in the flexor muscles than in the extensor muscles. Bradykinesia is characterized by decreased moving speed in combination with slower response in movement changes and reduced fine motor skills. Hypokinesia is defined as a decreased frequency and amplitude of the movements, such as reduced stride length, reduced arm swing etc. The typical stooped posture of PD patients is still absent in early PD and is caused by changes in body position and gait as a result of the increased rigidity (Mhyre et al., 2012).

This age-related disease has also been associated with other, non-motor symptoms: 30-40% of the PD patients are diagnosed with dementia, apathy, anxiety, psychosis and depression, 80-90% suffer from sleeping disorders and up to 90% of the PD patients display an altered olfactory perception (Mhyre et al., 2012). Autonomic disorders, including cardiovascular, gastrointestinal and genitourinary disorders, have also frequently been reported in PD patients. In 30-58% of the PD patients orthostatic hypotension can be detected as a result of cardiovascular autonomic dysfunction (Goldstein, 2006). Genitourinary disorders, such as bladder dysfunction, decreased libido and impaired sexual performances, have been described in 40-80% of the PD patients (Winge et al., 2006; Sakakibara et al., 2008; Mhyre et al., 2012). Upper gastrointestinal tract disorders, like oropharyngeal dysphagia and esophageal motility impairment, are generally present in up to 70% to 80% of the PD patients. A major complication of upper gastrointestinal tract dysfunction is food aspiration (25-30% of the patients), possibly resulting in aspiration pneumonia. Bloating, nausea and altered absorption of medication in PD patients is related to a delayed gastric emptying. Furthermore, compared to control patients the occurrence of infrequent bowel contractions, potentially leading to obstipation, megacolon and volvulvus, is clearly higher in patients suffering from PD. James Parkinson noted constipation as a feature in his essay (Parkinson 1817). In PD, frequency of defaecation diverges from that of controls three decades before the median age of neurological diagnosis (Charlett et al., 1997). Morphological and neurochemical changes of PD are found throughout the enteric nervous system and in dorsal vagal nuclei (Dobbs et al., 2008). Indeed, the presence of infrequent bowel movements is considered a risk factor for the development of PD (Abbott et al., 2001; Pfeiffer et al., 2003; 2011; Mhyre et al., 2012).

4.1.3 Incidence and prevalence

Studies in the US and Europe have reported a PD incidence between 12-19 cases/100.000 inhibitants per year (Twelves et al., 2003; Hirtz et al., 2007). Pringsheim and colleagues showed that the prevalence of PD in patients between 70 and 79 years old differed strongly depending on the geographical region: in North-America, Europe and Australia a prevalence of 1601 cases/100000 inhabitants has been reported, whereas a prevalence of 646 cases/100000 inhabitants has been reported in Asia. Furthermore, the same study observed that the prevalence of PD increased with age (Pringsheim et al., 2014). Another study reported that PD affects up to 1% of the population at 65-70 years of age, increasing to 4-5% of the population at the age of 85 (Fahn, 2003). Since age is considered the single greatest risk factor for the development of PD and the life expectancy of Western population is generally increasing, the number of PD cases is expected to increase (Dorsey et al., 2007). Compared to women, the prevalence of PD is 1.4 times higher in men (Twelves et al., 2003; Hirtz et al., 2007; Wirdefelt et al., 2011).

4.1.4 Etiology

Up to date the exact etiology of PD remains undetermined, however it is considered a multifactorial disease in which both genetic and environmental factors can play a role (Wirdefelt et al., 2011; Mhyre et al., 2012; Kannarkat et al., 2013). In 5-10% of the PD patients, the disease is the result of specific gene mutations, such as mutations in the α-synuclein-encoding gene (*PAK1/4, SNCA*), in the Leucin-Rich Repeat Kinase 2 (LRRK2-) encoding gene (*PARK8, LRRK2*), in the *parkin* (*PARK2*) locus and the Phosphatase and Tensin Homolog (PTEN)-induced Putative Kinase 1 (*PINK1*) encoding gene (Wirdefelt et al., 2011; Mhyre et al., 2012; Kannarkat et al., 2013). Mutations in the *α-synuclein* encoding gene and the *LRRK2* gene have been associated with dominantly inheritable parkinsonism, whereas mutations in *PINK* and *parkin* encoding genes have been associated with recessively inheritable parkinsonism (Tansey et al., 2007).

The majority of the PD cases are classified as sporadic/idiopathic Parkinsonism (IP), since the etiology remains unknown. However, different studies have identified several lifestyle and environmental risk factors for the development of IP. Generally, a negative association between smoking and coffee drinking on the one hand and the development of PD on the other hand has been reported (Wirdefelt et al., 2011; Mhyre et al., 2012). Additionally, a negative correlation between the occurrence of PD and physical activity, alcohol consumption and dietary intake of antioxidants has also been suggested. However, further research is recommended to confirm these associations. Besides, protracted pesticide exposure and the consumption of dairy products are considered as risk factors for the development of PD (Wirdefelt et al., 2011). Interestingly, some studies have mentioned the positive association between PD and rural living, well water consumption and farming. However, these findings were not confirmed by other researchers (Wirdefelt et al., 2011). This association was initially declared by the repeated protracted exposure to agrochemicals (Wirdefeldt et al., 2011). However, a study in 2002 showed that the mortality from PD among farmers from 26 US states was clearly elevated among live-stock farmers, but was lower than in non-farmers and arable farmers (Lee et al., 2002).

4.2 Pathogenesis of PD: dysfunction of neural barriers

In PD, neuro-inflammation is believed to contribute to neurodegeneration with activation of microglia, the residential macrophages of the central nervous system (Mhyre et al., 2012). Peripheral inflammation can activate microglia, in particular when the brain barriers are disturbed (Alvarez-Arellano et al., 2014). The brain is protected by four specialized brain barriers: the blood-brain barrier (BBB), the blood-cerebrospinal fluid barrier (blood-CSF barrier), the blood-retina barrier and the arachnoid barrier. These barriers determine what will (and what will not) enter the brain, and account for many physiological properties of their specific neural environments (De Bock et al., 2014). The focus in this PhD thesis lays on the BBB and the blood-CSF barrier. In the next section, information will be provided on these two neural barriers (BBB and the blood-CSF barrier) and their role in PD.

4.2.1 Blood-brain barrier

The BBB is located at the level of the capillaries of the CNS and is responsible for the regulation of the fluid exchange between the systemic blood circulation and the CNS. This neural barrier is formed by capillary endothelial cells and supported by surrounding astrocytes and pericytes (**Figure 8**), also referred to as the neurogliovascular unit (Ramsauer et al., 2002). Beside their supporting function of the endothelial cells, astrocytes play a role in the regulation of the expression of several tight junctions (Wolburg et al., 1994).

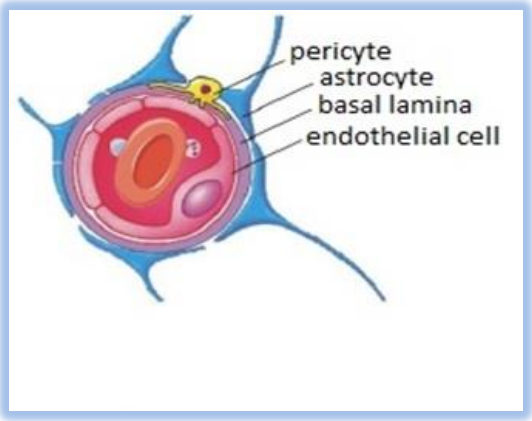

Figure 8 : Illustration of the BBB, consisting of capillary endothelial cells, astrocytes and pericytes (adapted from De Bock et al., 2014).

In order to prevent paracellular diffusion of different molecules, including toxic agents and cytokines from the systemic circulation to the brain, capillary endothelial cells are connected by tight junctions and adherens junctions (**Figure 9**) (Ek et al., 2006). Tight junctions, also referred to as zonulae occludentes (ZO), are formed by multiple transmembrane claudins in which occludin is incorporated (Furuse et al., 1998a, Furuse et al. , 1998b). Claudin-3, -5 and -12 play an important role in the connection between the capillary endothelial cells at the BBB (Nitta et al., 2003; Wolburg et al., 1994; Coisne et al., 2011). In contrast, occludin, which mainly has a supportive function, is not strictly necessary for the formation of the tight junctions (Furuse et al., 1998b; De

Bock et al., 2014). The tight junctions are mainly anchored to the actin (**Figure 9**) of the cytoskeleton of the endothelial cells by ZO proteins, ZO-1 and ZO-2 (De Bock et al., 2014). The adherence junctions are mainly formed by transmembrane cadherin proteins. This calcium-dependent adhesion molecule is responsible for cell-cell contact and it can be associated with other γ-catenines, such as β-catenine. In turn these molecules are connected with the cytoplasm of the cell by α-catenin (Vleminckx et al., 1999; Corada et al., 1999; Ek et al., 2006).

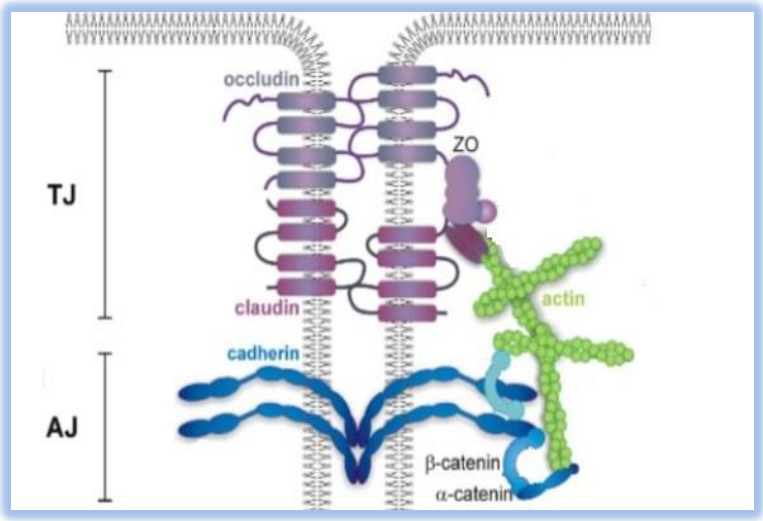

Figure 9 : Illustration of the tight junctions and adherens junctions of the blood-brain barrier. The tight junctions are formed by multiple claudins in which occludin is incorporated. In turn, occludin and claudin interact with the actin cytoskeleton via adaptor proteins, including the zonulae occludens proteins. The adherence junctions are formed by transmembrane proteins belonging to the cadherin family and are linked to actin via the catenins. ZO: zonulae occludens; TJ: tight junction; AJ: adherence junction (adapted from De Bock et al., 2014).

Disturbance of the BBB has been reported in patients suffering from PD (Stolp et al., 2009; Nielsen et al., 2012; Lee et al., 2014). Both local and systemic inflammation have been suggested to play a role in the increased permeability of the BBB. As a result of signaling through the endothelial cells, several locally or systemically produced cytokines, such as Il1-β, Il-6 and TNF-α, may cause alterations in the tight junction structure, thereby inducing an increased BBB permeability. The increased BBB permeability along with elevated levels of cytokines and inflammatory mediators, such as ROS, may cause neuro-inflammation and oxidative stress, leading to further BBB disruption and brain damage (Stolp et al., 2009; Lee et al., 2014). Furthermore, migration of white blood cells through the BBB as a result of the central inflammatory response has been suggested as well (Stolp et al., 2009).

4.2.2 Blood-cerebrospinal fluid barrier

The blood-CSF barrier is established by the single epithelial cell layer in the choroid plexus and is present in all four brain ventricles (Chodobski et al., 2001). The blood-CSF barrier is located between the CSF and the fenestrated capillaries of the choroid plexus (De Bock et al., 2014; Mortazovi et al., 2014). The cubical choroid plexus epithelial cells (CPEs) possess multiple microvilli and cilia at their apical side and a well perfused extracellular matrix, consisting of connective tissue in combination with collagen, fibroblasts, macrophages and

dendritic cells, at their basal side (**Figure 10**) (Dohrmann et al., 1970; Mortazavi et al., 2014). They are the main producers of CSF (Cushing, 1914) and the remaining 10-30% of the CSF volume originates from the interstitial fluid in the brain (Cserr et al., 1986). The CSF is responsible for the transportation of necessary nutrients and other components in the brain by migrating from the lateral ventricles through the 3th and 4th ventricle to the subarachnoid space of the brain (Chodobski et al., 2001). Additionally, the choroid plexus participates in the elimination of harmful substances and degradation products in the CSF (Coisne et al., 2011) and plays an important role in the communication between the peripheral immune system and the CNS. Indeed, after leakage from the fenestrated capillaries, B cells, T cells and monocytes are able to migrate from the choroid plexus into the brain via specific transport mechanisms (Provencio et al., 2005; Meeker et al., 2012; Demeestere et al., 2015). Inflammation has already been linked with increases in the transport of these immune cells, resulting in a higher number of immune cells in the CSF (Vercellino et al., 2008). Furthermore, an upregulation of the expression of cytokines and inflammatory mediators has been reported in case of systemic inflammation (Kim et al., 2012; Vandenbroucke et al., 2012). Indeed, there is evidence that peripheral/systemic inflammation influences the pathophysiology of PD. In PD patients, there are gradients of objective measures of PD facets on serum cortisol and TNF-α, and of clinical motor scores on peripheral blood mononuclear cell production of cytokines and NF-κB expression (Charlett et al. 1998; Dobbs et al. 1999; Reale et al, 2009). Serum cortisol is elevated (17%) in PD, as is IL-6. Moreover, in a prospective study, a higher concentration of IL-6, in blood collected 4 years previously, predicted incident PD (Chen et al. 2008). There are also gradients of objective measures of facets of PD on blood leucocyte subtypes counts (Dobbs et al, 2012). Both brady/hypokinesia and flexor rigidity are worse the higher the NK count.

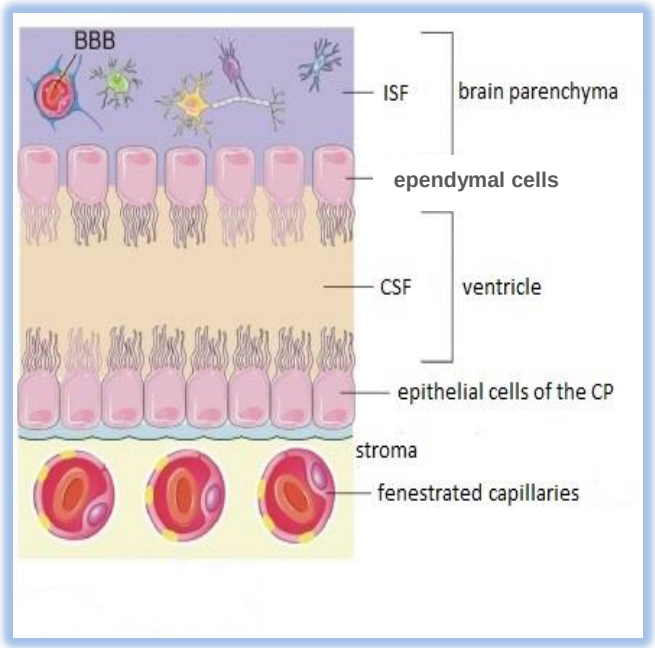

Figure 10 : Illustration of the blood-cerebrospinal fluid barrier formed by the choroid plexus epithelial cells. These cells form a barrier between the CSF and the fenestrated capillaries of the stroma of the choroid plexus and they are localized in all four brain ventricles. CP: choroid plexus; ISF: interstitial fluid; CSF: cerebrospinal fluid; BBB: blood-brain barrier (adapted from De Bock et al., 2014).

Like the endothelial cells in the BBB, the CPEs are inter connected by a network of tight junctions. Together they allow the strict regulation of transport through the blood-CSF barrier (De Bock et al., 2014). Whereas claudin-3, -5 and -12 are important in the BBB, the main tight junction proteins expressed in the blood-CSF barrier are claudin-1, -2, -3 and -11. Claudin-2 in necessary for the formation of paracellular water channels (Rosenthal et al., 2010; Kratzer et al., 2012). As for the BBB, the transmembrane claudins are connected to the cytoskelet of the cells via the ZO-1 and ZO-2 proteins (De Bock et al., 2014). Since the barrier formed by the choroid plexus cells is less tight compared to the BBB, the blood-CSF barrier is more susceptible to systemic alterations and inflammation and thereby this neural barrier is strongly subjected to immunologic influences (De Bock et al., 2014).

PD has also been associated with an increased permeability of the blood-CSF barrier (Vawter et al., 1996; Pisani et al., 2012). Significantly elevated levels of transforming growth factor beta (TGF-β) were demonstrated in the CSF of PD patients compared to controls. The same study suggested that some of the TGF-β in the CSF emanated from the plasma as a result of leakage (Vawter et al., 1996). Another study reported a significant increase in the albumin ratio (CSF albumin/serum albumin) in advanced-stage PD patients, compared to early-stage PD patients and healthy controls (Pisani et al., 2012).

4.3 Involvement of a *H. pylori* infection in PD: what is known so far?

As already mentioned above, the association between PD and gastrointestinal complaints has been suggested. Constipation, resulting from infrequent bowel movements and slow gut-transit (Abbott et al., 2001; Dobbs et al., 2016), has been described as one of the most common co-morbidities in PD patients (Parkinson, 1817; Dobbs et al., 2016). Schwab and colleagues were the first to propose the association between PD and the presence of gastric and duodenal ulcers (Schwab et al., 1961). In 1965, this was confirmed by Strang who reported an excess of previously diagnosed peptic ulcers in PD (Strang, 1965). Subsequently, the speculation that an infectious agent might be involved in both PD and peptic ulcers was suggested (Szabo, 1979). After the discovery of *H. pylori* in the stomach of human patients suffering from gastritis and peptic ulcers in 1984 (Warren and Marshall, 1984), Altschuler and collegues were the first to explicitly suggest the causal link between PD and *H. pylori* (Altschuler et al., 1996). Ever since, the association between IP and an infection with *H. pylori* has been described by multiple other authors (Dukowicz et al., 2007; Nielsen et al., 2012; Dobbs et al., 2008; Dobbs et al., 2012; Tan et al., 2015; Csoti et al., 2016; Dobbs et al., 2016; Tan et al., 2015).

Up to date, a high prevalence of *H. pylori* infections has been reported in PD patients (Rees et al., 2011; Nielsen et al., 2012). Additionally, *H. pylori* seropositivity was 3 times more likely to occur among PD patients compared to control patients (Brady et al., 1998; Charlett et al., 1999). The same study showed that, compared to a control group, siblings of PD patients were more likely to be *H. pylori* seropositive and present Parkinson-like symptoms, such as brady/hypokinesia of gait, bradykinesia of hands, abnormal posture and rigidity (Brady et al., 1998; Charlett et al., 1999). Furthermore, compared to age-matched control patients, an increase of the level of *H. pylori* antibodies was reported in a group of patients (younger than 80 years) displaying Parkinson-

like symptoms (Dobbs et al., 2000). Another study described that the odds of parkinsonism increased fivefold (up to the age of 80) in case of CagA-positivity in combination with VacA negativity and urease B negativity (Weller et al., 2005). Moreover, increased numbers of natural killer cells have recently been associated with shorter stride, slower gait and elevated flexor rigidity in patients suffering from PD (Dobbs et al., 2012). Interestingly, the same study described higher numbers of natural killer cells in case of urea-breath test positivity as well as additional reduction in stride and speed in *H. pylori*-positive PD patients (Dobbs et al., 2012).

H. pylori eradication therapy, consisting of 20 mg omeprazole, 500 mg clarithromycin and 1 g amoxicillin, resulted in the improvement of the stride length in PD patients (Bjarnason et al., 2005; Dobbs et al., 2010). Despite a worsening of the upper limb flexor rigidity, an overall improvement of PD-related symptoms, irrespective of anti-parkinsonian medication, could be observed after biopsy-proven successful eradication of *H. pylori* in PD patients (Dobbs et al., 2010). Clarithromycin and/or amoxicillin were replaced by administration of 500 mg tetracycline and/or 400 mg metronidazole in case of *in vitro* resistance of *H. pylori* isolates (Dobbs et al., 2010; Dobbs et al., 2013).

Furthermore, *H. pylori* eradication therapy in patients suffering from PD resulted in an improved response to the administration of L-3,4-dihydroxyphenylalanine (levadopa) in combination with an amelioration of motor symptoms and life quality. Levadopa, which is mainly absorbed in the duodenum, is used as treatment of PD, as it is a precursor of dopamine. Since *H. pylori* infections may induce a disruption of the gastrointestinal mucosa (Hashim et al., 2014; Caron et al., 2015), an infection with this bacterium may thus interfere with the absorption of levadopa, resulting in decreased plasma levels of levodopa and thereby in a faster progression of PD-related symptoms (Pierantozzi et al., 2001; Fasano et al., 2013; Hashim et al., 2014). However, Dobbs et al. (2005) did not find any major differences in hypokinesia improvement following biopsy-proven *H. pylori* eradication between PD patients receiving levadopa and PD patients who were never treated with levodopa, suggesting that the effect on hypokinesia was not due to improved absorption of levodopa as a result of *H. pylori* eradication.

Several hypotheses have been proposed to declare the association between PD and *H. pylori*: it has been suggested that a chronic infection with *H. pylori* results in acquired immune suppression by the continuous antigenic stimulation from *H. pylori* infection in combination with increased circulating IL-6, thereby leading to autoimmunity (Dobbs et al., 2000). Another hypothesis is that there might be molecular mimicry between antigens from humans and *H. pylori.* Possible candidates are the heat shock proteins (hsp), which appear to be highly conserved from bacteria to mammals. Indeed, hsp60 antibodies have been detected in both *H. pylori*-positive patients and PD patients (Dobbs et al., 2000).

In conclusion, these data suggest that gastric infections with *H. pylori* might be associated with PD. In contrast, no information is currently available on whether there might be a possible link between PD and *H. suis* infections in human patients. Further research is required to obtain more information on the role of gastric *Helicobacter* infections in PD.

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SCIENTIFIC AIMS

Helicobacter pylori (*H. pylori*) is considered to be the most important pathogen involved in gastric disease in humans. Besides causing inflammation and lesions in the stomach, infection with this agent has also been linked to extragastric manifestations, including brain disorders (Holtmann and Talley, 2014). Gastric pathologies in humans have, however, also been associated with spiral-shaped, gastric *Helicobacter* species different from *H. pylori* (non-*H. pylori* helicobacters (NHPH)). *H. suis*, an organism with a corkscrew-like morphology that naturally colonizes the stomach of pigs and non-human primates, is considered to be the most prevalent NHPH in humans. The role of gastric bacteria, other than *H. pylori,* in brain functionality is largely unknown. There is, however, a case report suggesting that infections with *H. suis* might also be associated with Parkinson's disease (PD) (Dobbs et al., 2005).

Therefore, the **general aim** of this thesis was to evaluate if and how a gastric infection with *H. suis* might be linked to PD in human patients.
Since it became clear during these studies that the current diagnostic tests used for detection of *H. suis* infections suffered from severe shortcomings, an **additional aim** of the thesis was to improve diagnostic assays.

It has been reported that an *H. pylori* infection might be associated with PD. Overall in idiopathic parkinsonism (IP), successful *H. pylori* eradication was shown to improve brady/hypokinesia. A poorer response was seen in the anti-nuclear antibody seropositive, who may have had residual *Helicobacter*, undetected by culture and PCR. Overt failure was accompanied by marked deterioration. Apart from *H. pylori* infections, IP has also been associated with rural-living and farm-experience, especially live-stock farm-experience. To determine whether there might be an association between PD and *H. suis* infections in humans, the **first specific aim** of this thesis was to compare the prevalence of *H. suis* in gastric biopsies between patients suffering from IP and controls.

In our first study we showed that the frequency of *H. suis* was significantly higher in IP patients compared to control patients. However, it remained unclear if an infection with this gastric microorganism might play a role in aethiopathogenesis. The brain is protected from alterations in the systemic circulation by several specialized neuro-epithelial barriers, such as the blood-brain barrier and the blood-cerebrospinal fluid (CSF) barrier, that determine what will (and what will not) enter the brain. Dysfunction of these brain barriers has been implicated in the pathogenesis of PD. It has been suggested that systemic inflammation could play an important role in the onset of neurodegenerative disorders: it might be accompanied by barrier disruption associated with immune/inflammatory mediators. Since gastric *Helicobacter* infections have been proposed to be associated with systemic inflammation, the **second specific aim** of this thesis was to evaluate whether and how a *H. suis* infection could have an impact on the brain homeostasis.

In order to determine the importance of an *H. suis* infection in gastric and extragastric disorders in human patients, accurate diagnostic methods for the specific detection of *H. suis* in gastric biopsies are required. Currently, the diagnosis of infections with this spiral-shaped bacterium is hampered by its fastidious nature, the low colonization density and the patchy distribution of *H. suis* in the human stomach. Additionally, when using

the conventional *ureA*-based SYBR green RT-PCR for the detection of *H. suis* infections, non-specific interactions with host DNA and DNA from other *Helicobacter* spp. may occur, as was noticed during our first study. Thus, **the third specific aim** of this thesis was to optimize the currently used methods for demonstration of *H. suis* in the stomach. We first developed and validated a new, probe-based RT-PCR that allowed detection and quantification of *H. suis* in gastric tissue samples. Secondly, a mouse-passage-based protocol was optimized for the isolation of low numbers of *H. suis* bacteria.

EXPERIMENTAL STUDIES

Chapter 1

Significantly higher frequency of *Helicobacter suis* in patients with idiopathic parkinsonism than in control patients

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Abstract

Background: There is increased proportional mortality from Parkinson's disease (PD) amongst livestock farmers. The hypokinesia of Parkinson's disease has been linked to *Helicobacter pylori* (*H. pylori*). *Helicobacter suis* (*H. suis*) is the most common zoonotic *Helicobacter* in man.

Aims: To compare the frequency of *H. suis*, relative to *H. pylori*, in gastric biopsies of patients with idiopathic parkinsonism (IP) and controls from gastroenterology services.

Methods: Coded DNA extracts, archived at a *Helicobacter* Reference Laboratory, from IP patient and gastroenterology service biopsies were examined for *H. suis*, using species-specific RT-PCR.

Results: Relative risk of having *H. suis* in 60 IP patients compared with 256 controls was 10 times greater than that of having *H. pylori*. In patients with IP and controls, respectively, frequencies of *H. suis* were 27 (exact binomial 95% C.I. 15, 38) and 2 (0, 3)%, and of *H. pylori*, 28 (17, 40) and 16 (12, 21)%. Excess of *H. suis* in IP held when only the antral or corporal biopsy was considered. Of 16 IP patients with *H. suis*, 11 were from 19 with proven *H. pylori* eradication, 3 from 17 pre-*H. pylori* eradication, 2 from 24 *H. pylori* culture/PCR negative. Frequency was different between groups (P = 0.001), greatest where *H. pylori* had been eradicated. Even without known exposure to anti-*H. pylori* therapy, *H. suis* was more frequent in IP patients (5/41) than in controls (1/ 155) (P = 0.002). Partial multilocus sequence typing confirmed that strains from IP patients (6) and control (1) differed from RT-PCR standard strain.

Conclusions: Greater frequency of *H. suis* in idiopathic parkinsonism appears exaggerated following *H. pylori* eradication. Multilocus sequence testing comparison with porcine strains may clarify whether transmission is from pigs/ porcine products or of human-adapted, *H. suis*-like, bacteria.

INTRODUCTION

The broad brush of epidemiology has provided enigmatic aetiopathogenic clues in Parkinson's disease (PD). It has been linked, albeit inconsistently, with rural living and farm experience (Wirdefeldt et al., 2011). Such associations have long been explained away by putative exposure to agrochemicals. However, a mortality study in 26 US states found increased proportional mortality from PD among livestock, but not arable, farmers (Lee et al., 2002). From a total of 6 million death certificates, 267479 decedents were classified as either crop or livestock farmers. Amongst white males, the proportional mortality from PD was significantly higher in livestock farmers than in non-farmers, but lower in crop farmers. Zoonotic infections were considered as an explanation, but it was noted that the pesticides used in animal infestations are different from those used on crops. These effects were not replicated in the much smaller numbers of White female or of African American decedents.

The 1965 finding of prodromal peptic ulcers in PD (Strang, 1965) paved the way to exploring any link between (the then undiscovered) *Helicobacter pylori* (*H. pylori*) and PD (Dobbs et al., 2008; 2012; 2010; 2013). The 2012 Maastricht IV Consensus Report (Malfertheiner et al., 2012) acknowledges interesting associations. In a randomised placebo-controlled trial, we found that biopsy-proven *H. pylori* eradication had differential effects on objective measures of PD facets: improvement in hypokinesia and worsening rigidity over the year post-eradication, both plateauing over the next year (Dobbs et al., 2010). Overall, there was clinically relevant improvement. This was independent of any (stable, long t½) anti-parkinsonian medication. Receipt of levodopa was an exclusion. Moreover, the effect on hypokinesia was indication-specific (Dobbs et al., 2013). A longitudinal observational study confirmed improvement in hypokinesia following *H. pylori* eradication. Anti-microbials for other indications had no such effect, but successive courses were associated with cumulative increase in rigidity.

Could, then, zoonotic-transmission of gastric non-*H. pylori Helicobacter* species (NHPH) contribute to PD? The term NHPH represents a group of closely related, but distinct, bacterial species found in different animal species, such as *H. suis* in pigs, and *H. felis*, *H. salomonis*, *H. bizzozeronii*, *H. heilmannii* sensu stricto, *H. cynogastricus* and *H. baculiformis* in cats and dogs (Haesebrouck et al., 2009). Although infection with these bacteria has been associated with human gastric disease in a substantial number of patients (Debongnie et al., 1995; Morgner et al., 1995; Stolte et al., 1997; Trebesius et al., 2001; O'Rourke et al., 2004; Van den Bulck et al., 2005; Joosten et al., 2013), human NHPH strains have been isolated on only three occasions (Andersen et al., 1996; Kivisto et al., 2010; Wüppenhorst et al., 2013). Subsequent identification to species level has revealed these to be *H. bizzozeronii* in two cases (Kivisto et al., 2010; Javala et al., 2001) and *H. felis* in the other (Wüppenhorst et al., 2013). Based on histopathology of gastric biopsy, the prevalence of NHPH in patients with gastric disease has been estimated at between 0.2% and 6%, depending on geographical distribution (Stolte et al., 1997). Several studies have demonstrated that *H. suis* is the most frequent NHPH species in humans (Haesebrouck et al., 2009; Trebesius et al., 2001; Van den Bulck et al., 2005). However, its *in vitro* culture, starting from human gastric mucosa, has been unsuccessful so far, underlining the extreme fastidiousness of gastric NHPH in general and *H. suis* in particular.

We report the frequency of *H. suis* DNA in archived DNA extracts from gastric biopsies in a group of PD patients and a group of gastroenterology patients, as part of a service evaluation. The relative prevalence of *H. pylori*

immunoblot seropositivity in people with and without PD (37% and 31% respectively) has been reported (Charlett et al., 2009). Here, we standardise the frequency of *H. suis* positivity against that recorded for *H. pylori* (culture, or if negative, PCR), in order to compare the two sets of archived DNA extracts.

METHODS

Service evaluation

The term '*H. heilmannii*' was commonly used to describe spiral gastric helicobacters seen on histopathology. The diagnostic service of the Gastrointestinal Reference Unit, Public Health England, for PCR detection of '*H. heilmannii*-like organisms' in gastric biopsy material (Chisholm et al., 2003) was re-evaluated, using coded archived DNA extracts. As *H. suis* is the commonest reported NHPH in humans and a species-specific assay is now available (O'Rourke et al., 2004), it is a good starting point for evaluating whether NHPH cases are being missed. Interestingly, the original '*H. heilmannii*' assay was set up in response to a case of a spiral *Helicobacter* in PD (Dobbs et al., 2005).

Sourcing coded DNA extracts

The *Helicobacter* Reference Laboratory in the Gastrointestinal Bacteriology Reference Unit, Public Health England, held DNA from gastric biopsies, extracted (at time of receipt) over the last decade, stored at -80 °C. Biopsies had been received with request for *Helicobacter* culture (isolation/identification/anti-microbial susceptibility) and, if biopsy culture-negative, detection of *H. pylori*-specific DNA. Culture-negative biopsies had been tested using a PCR targeting *16S rRNA* (primer pair HP1⁄HP2) (Ho et al., 1991) and *vacA* (Vac3624F⁄Vac3853R) (Chisholm et al., 2001) genes. To detect '*Helicobacter heilmannii*-like organisms' a 16S rRNA assay had been used (Chisholm et al., 2003). For the service evaluation, archived DNA extracts, identified only by their accession number, were couriered to the Laboratory of Veterinary Bacteriology and Mycology, Ghent University. All extracts archived over this decade (paired antral and corporal biopsies) from 60 patients with 'clinically definite' idiopathic parkinsonism (IP) (Calne et al., 1992), diagnosed at a National clinic, were retrieved. Clinically definite IP refers to any combination of three of the four cardinal features: resting tremor, rigidity, brady/hypokinesia, impaired postural reflexes. Alternatively sufficient is having two of the features, with one of first three asymmetrical (Responsiveness to a dopaminergic drug challenge was not a requirement). Details of anti-microbials used in any previous anti-*H. pylori* treatment (19/60) had been recorded on the request form.

DNA extracts from biopsies originating from English gastroenterological services, selected for attention to documenting any exposure to anti-*Helicobacter* therapy on the request form, were used as 'controls'. This criterion yielded extracts from 256 patients, archived over a similar period (single biopsy on given occasion except in four, where two sites sampled). There had been previous exposure to anti-*Helicobacter* therapy in 101, no recorded exposure in the rest. No request form mentioned Parkinson's disease, or any other issues outside the context of diagnostic endoscopy. Crude prevalence rate estimates for PD in 34 European studies range widely, from 65.6 to 12500 per 100000 (von Campenhausen et al., 2005): that is, 0–32 diagnosed cases would be expected in the gastroenterological services' patient group, even if they had no particular predisposition.

Frequencies of *Helicobacter* species reported here are not construed as prevalence: there is selection in the controls (for adequate documentation by above criterion) and of IP patients (for a clinic with particular interest in the gastroenterological aspects of PD). Any direct estimate of association between presence of *H. suis* and PD could be biased by cohort selection. We therefore obtain an estimate of the risk of *H. suis* in patients with IP compared with those from gastroenterological services by standardizing for the relative risk of having *H. pylori*, and by setting *H. suis* frequency in context of exposure to anti-*Helicobacter* therapy. No analysis of the relationship of *H. suis* status to gastric symptoms was planned: the request form did not contain a checklist.

***H. suis* detection**

The presence of *H. suis* DNA in the extracts was determined using a *H. suis*-specific quantitative real time (RT)-PCR, based on the *ureA* gene. For generating the standard, part of the *ureAB* gene cluster (1236 bp) from *H. suis* strain HS5 was amplified using primers U430F and U1735R, as described previously (O'Rourke et al., 2004). The standard consisted of 10-fold dilutions, starting at 10^8 PCR amplicons, for each 9 µL of reaction mixture. One microliter of extracted DNA template was added to 9 µL reaction mixture, consisting of 0.25 µL of both primers located within the 1236 bp fragment (to yield a 150 bp PCR product), 3.5 µL HPLC water and 5 µL SensiMixTM SYBR No-ROX (Bioline Reagents Ltd, London, UK). Sense primer was BF_HsuisF1: 5'-AAA ACA MAG GCG ATC GCC CTG TA-3'. Anti-sense primer was BF_HsuisR1: 5'-TTT CTT CGC CAG GTT CAA AGC G-3'. Annealing temperature was 62 °C. Both standards and samples were run in duplicate on a CFX96TM RT-PCR System with a C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA). To confirm the presence of *H. suis* DNA, all positive samples were sequenced (Joosten et al., 2013). All 413 available extracts (including replicate biopsies at a given time and any follow-up biopsies) were assayed on two separate occasions to estimate between-assay agreement. To demonstrate that the *H. suis* DNA in the biopsy extracts was different from the *H. suis* strain HS5 DNA used as standard in the RT-PCR, multilocus sequence typing (MLST) was performed with seven housekeeping genes, as previously described (Liang et al., 2013).

Statistical analysis

Use of exact binomial confidence intervals provided an estimate of the uncertainty in estimates of proportions. The risk ratio, risk of having *H. suis* in IP patients to that in controls compared with relative risk of having *H. pylori*, was estimated from the paired data on the two species, using a conditional Poisson regression with robust standard errors (Cummings, 2011).

RESULTS

The distribution of age at time of first biopsy in controls [mean 52 (data interval 24, 81) years] encapsulated that in the IP patients [62 (45, 79) years]. There was no significant difference in the proportion of male patients: 57% of the IP patients were male (34/60), 47% (120/256) of the controls. Overall agreement (99.3%) between the two *H. suis* RT-PCR assay runs on separate occasions was strong (n = 413, Kappa = 0.95, P < 0.001: null hypothesis of no agreement rejected) and there was no significant difference between the first and second run {exact McNemar's test, ratio of paired proportions 1.11 [95% confidence interval (CI) 0.99, 1.24], P = 0.25}. Thirty-one of the 413 extracts were positive in either run, 28 in both. Nucleotide sequencing of positive samples, and subsequent Basic Local Alignment Search Tool (BLAST) analysis, revealed 95–100% homology with

known *H. suis* strains. The frequency of detection *of H. suis* and *H. pylori* in the extract(s), held in the archive from the 60 IP patients and 256 controls, is shown in Table 1. In patients biopsied on more than one occasion, only the first occasion is considered. Overall, *H. suis* DNA was present in 27 (binomial exact 95% CI 15, 38)% of the IP patients and in 2 (0, 3)% of controls. It was present in 18 (10, 30)% of antral biopsies and 13 (6, 25)% of corporal biopsies from IP patients. Thus, the excess *H. suis* frequency was not an artefact due to sampling both regions in IP, but only one region (46% antral, 1% corporal, 2% duodenal, rest 'gastric') in all but four controls (2 had antral and corporal biopsies, 1 antral and duodenal, in 1 both were labelled 'gastric').

Table 1: Frequency of *H. suis* contrasted with *H. pylori* in gastric biopsy DNA extracts from IP patients and controls with and without known exposure to anti-*H. pylori* therapy.

Frequency	IP patients % (no. with species/total)	Controls % (no. with species/total)
H. suis		
Previous exposure to anti-*H. pylori* therapy	58 (11/19)*	3 (3/101)
No known exposure	12 (5/41)#	1 (1/155)
Total	27 (16/60)**	2 (4/256)**
H. pylori		
Previous exposure to anti-*H. pylori* therapy	0 (0/19)	22 (22/101)
No known exposure	41 (17/41)	13 (20/155)
Total	28 (17/60)	16 (42/256)

*: Anti-microbial prescription was guided by *in vitro* sensitivities:- Of the 11 with *H. suis*, 10 had received 1 week of amoxicillin, clarithromycin and proton pump inhibitor (PPI) (1 a further course of metronidazole, tetracycline, PPI and tripotassium dicitratobismuthate when he remained urea breath test-positive) and 1 had received 1 week of clarithromycin, tetracycline and PPI. Of 8 without *H. suis*, 7 had received 1 week of amoxicillin, clarithromycin and PPI and 1 had received 1 week of clarithromycin, tetracycline and PPI.

#: Three extracts also positive for *H. pylori*.

**: Mean age (gender) of the 16 IP patients with *H. suis* was 58 (range 47–68) years (10 male, 6 female), of the 4 controls, 54 (43–64) years (1 male, 3 female).

The relative risk of having *H. suis* in IP patients compared with controls was 9.9 times greater than that of having *H. pylori* {i.e. [(16/60)/(4/256)]/[(17/60)/(42/256)] = 17.07/1.73}. The 95% confidence interval (3.0, 32.7) did not include 1, indicating that the two relative risks are significantly different. This was despite a higher frequency of *H. pylori* in IP patients [28 (binomial exact 95% C.I. 17, 40)%] than in controls [16 (12, 21)%]. Of the 16 IP patients with *H. suis*, 11 were from the 19 with proven H. *pylori* eradication, 3 from the 17 pre-*H. pylori* eradication and 2 from the 24 who were *H. pylori*-negative. Frequency of *H. suis* detection differed significantly among these three groups (Fisher's exact test, P = 0.001), being greatest in those who had undergone anti-*H. pylori* therapy than in the remainder. There was no significant difference in the anti-*H. pylori* regimen

(clarithromycin/amoxicillin/proton pump inhibitor or other: footnote Table 1) between those with and without *H. suis* in its aftermath. Even in those without known previous exposure to anti-*H. pylori* therapy, *H. suis* was more frequent in IP patients (5/41) than in controls (1/155) (P = 0.002). The low frequency of *H. suis* in the 256 controls precluded estimation of any increased risk with exposure to anti-*H. pylori* therapy. Partial MLST results for the *H. suis* DNA found in extracts from 6 IP patients and 1 control are shown in Table 2. The allele pattern in these samples differed from that in the HS5 DNA standard: that is, the sample results were not due to contamination. It was not possible to obtain complete MLST sequence types, due to interference from human DNA and the low amount of *H. suis* DNA found in some extracts. None of the samples had been reported as positive for '*Helicobacter heilmannii*-like organisms' on the original assay 16S rDNA assay.

Table 2: Partial *H. suis* MLST results on DNA extracts from gastric biopsies of IP patients and a control

	Allele no.						
DNA extract	***atpA***	***efp***	***mutY***	***ppa***	***trpC***	***ureAB***	***yphC***
IP patient 1	4		11				
IP patient 2		1					
IP patient 3						1	
IP patient 4		1	11				
IP patient 5			5			1	1
IP patient 6		1	5				
Control patient		1	1			1	
HS5*	4	2	4	1	1	1	1

*: positive control in the quantitative RT-PCR assays.

DISCUSSION

Relative frequency of *H. suis* in IP

The frequency of finding *H. suis* DNA in extracts from gastric biopsies in a well-defined disease state, clinically definite IP, contrasts sharply with the apparently 'sporadic' nature in patients from gastroenterological services undergoing diagnostic endoscopy. The statistical significance of the finding withstood standardization for the relative frequency of *H. pylori* in the two patient groups. However, defining the strength of association between presence of *H. suis* DNA in gastric biopsies and PD will clearly require representative cohorts of IP probands and controls. As human infection with NHPH is characteristically sparse and patchy (Stolte et al., 1997), we may be underestimating the true frequency of *H. suis* in both patient groups. There are no previous studies of the frequency of NHPH in PD for comparison.

Proposed pathophysiological role of *Helicobacter* species in IP

The concept of 'brain-altering' remote infections is receiving increasing attention. Neuro-inflammation in IP may not merely be the adverse effect of microglial scavenging of degenerating neurons and reaction to aberrant protein (Tansey et al., 2007; Zipp et al., 2006). If PD is driven by systemic immuno-inflammatory processes (Dobbs et al., 2008; 2012), intervention against them, or their driving forces, could modify its course

(Dobbs et al., 2013; Malfertheiner et al., 2012). A U-turn in brady/hypokinesia and weight gain was described after eradicating a spiral *Helicobacter*, associated with antral gastritis, in a cachectic IP patient, who had been wheelchair-bound without assistance for over a year (Dobbs et al., 2005). The aetiopathogenic significance of zoonotic helicobacters in IP remains to be explored. Finding clinical correlates (e.g. epidemiological, such as mortality, and pathophysiological, such as circulating inflammatory markers) would suggest that *H. suis* DNA in gastric mucosa is of significance in IP. Autoimmunity is suggested as the mechanism of the effect of *H. pylori* eradication on hypokinesia (Dobbs et al., 2008; 2010; 2012). Response appears unrelated to infection load. Poor response is associated with anti-nuclear antibody seropositivity. There are HLA-DR risk loci for PD. If NHPH eradication has a similar effect, then the immune hypothesis could move to a pattern recognition response rather than classical HLA-restricted autoimmunity.

Optimizing diagnosis of human *H. suis* infection

Diagnosing NHPH in IP is problematic. Our experience is that the urea breath test is usually negative, except where *H. pylori* co-exists. A serological test for *H. suis* would provide a useful screen for infection, or any memory of it, in potentially high-risk patient or occupational groups, but none is currently available. The 16S rDNA assay for '*Helicobacter heilmannii*-like organisms' (Chisholm et al., 2003) did not detect the *H. suis* found on RT-PCR. It may, in part, be a question of sensitivity. *Helicobacter heilmannii*-like DNA (Chisholm et al., 2003) had been detected in a urea breath test-positive case (Dobbs et al., 2005): re-evaluation by the methodology used here showed this to *be H. suis*. There appears to be advantage in taking more than one biopsy to determine NHPH status by molecular microbiology. Low infection load (Stolte et al., 1997) will hamper histological confirmation. Sensitivity and specificity of the RT-PCR will need to be determined for introduction into routine clinical use. This is not as problematic for *H. suis* as for other NHPH, since it is the only gastric *Helicobacter* isolated from pigs in Europe, where infection is very common and load high. The gold standard of seeing spiral gastric helicobacters, in a histological section adjacent to the biopsy for DNA extraction, can be applied. Greater frequency of *H. suis* in IP where *H. pylori* has been eradicated points to need for post-treatment endoscopic biopsy. New infection in a particularly susceptible host cannot be excluded, although adult transmission of another gastric *Helicobacter, H. pylori*, is unusual. It is likely that the IP patients were co-infected with *H. pylori* and *H. suis* prior to eradicating *H. pylori*, and *H. suis* filled the niche afterwards. Indeed, susceptibility testing of isolates from sows suggests relative intrinsic insensitivity to amoxicillin and metronidazole, greater intrinsic susceptibility to tetracycline (Vermoote et al., 2011). Current use of tetracycline in first-line treatment strategies is limited (Malfertheiner et al., 2012). Information on *in vitro* anti-microbial susceptibility of *H. suis* in man is needed.

Transmission of *H. suis*

Contact with pigs is a risk factor for human gastric *H. suis* infection (Meining et al.,1998). It was demonstrated, using MLST, that a *H. suis* strain from the stomach of a pig veterinarian with gastric complaints was closely related to porcine strains (Joosten et al., 2013). This illustrates the zoonotic potential of this species: direct contact with pigs can be a source for human *H. suis* infection. Moreover, *H. suis* can be present, and survive, in minced pork (De Cooman et al., 2013): raw or undercooked porcine products might be another source of infection. Complete MLST sequence types were not obtained for any of the *H. suis* strains from patients,

precluding comparison with sequence types present in pigs. Although all alleles of the housekeeping genes detected in patients have been found in pig *H. suis* strains (Liang et al., 2013), the patients' strains could be *H. suis*-like, human-adapted bacteria. Isolation of strains from patients' stomachs could throw light on their origin. Challenges are the fastidious nature of *H. suis*, and obtaining fresh human biopsies from infected mucosa, where infection is not confluent and biopsies are small, non-targeted and limited in number (Haesebrouck et al., 2009). Horizontal and/or vertical transmission of NHPH might contribute to explaining the epidemiology of IP. Whilst we do not know if NHPH can be passed between humans, most *H. pylori* infections are transmitted where there is close contact, as between parent or sibling and infant. This fits with the siblings of IP patients sharing facets of the syndrome and increased frequency of *H. pylori* seropositivity (Dobbs et al., 2008). Adult transmission of a causative factor is suggested by spouses of IP patients being a short, but highly significant, 'distance-down-the-pathway' (Dobbs et al., 2008), with marked, multifarious, relevant differences between spouses and control couples. These are difficult to explain by selective mating or learnt/reactive behavior.

Future directions

Irrespective of association of *H. suis* with PD, its known association with gastric pathology requires its detection to be added to diagnostic microbiology on gastric biopsies from patients in high-risk groups. Feedback of results of the service evaluation to individual IP patients, who were traceable by the single clinic, has been made, with the option of re-biopsy in those with historical *H. suis* infection. A natural progression of the work is to examine the archived samples for zoonotic helicobacters commonly associated with cats and dogs.

AUTHORSHIP

Guarantor of the article: Dr Sylvia Dobbs. Author contributions: RJD, SMD, IB and AL requested FH's expertise, in re-evaluating the NHPH assay used at Public Health England. FH, RJD and SMD were joint principal investigators. CB & AS contributed equally. CB, AS, BF, AL performed the laboratory work. CB, AS, AL, FH, SMD, RJD, AC collected and analyzed the data. AS, AL, AC, RJD, SMD, FH designed the study. CB, BF, FP, RD, DT, CW, IB contributed to the design. CB, AS, FH, IB, AC, SMD, RJD wrote the paper. All authors have approved the final version of the manuscript.

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Chapter 2

The choroid plexus epithelium as a novel player in the stomach-brain axis during *Helicobacter* infection

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Abstract

Several studies suggest a link between shifts in gut microbiota and neurological disorders. Recently, we reported a high prevalence of *Helicobacter suis* in patients with Parkinson's disease. Here, we evaluated the effect of acute gastric *H. suis* infection on the brain in mice. One month of infection with *H. suis* resulted in increased brain inflammation, reflected in activated microglia. Two communication pathways between the gastrointestinal tract and the brain were shown to be involved. We detected choroid plexus inflammation and disruption of the epithelial blood-cerebrospinal fluid (CSF) barrier upon *H. suis* infection, while the endothelial blood-brain barrier (BBB) remained functional. These changes were accompanied by leakage of the gastrointestinal barrier and low-grade systemic inflammation, suggesting that *H. suis*-evoked gastrointestinal permeability and subsequent peripheral inflammation induces changes in brain homeostasis. Additionally, *H. suis* infection caused changes in the signaling of vasoactive intestinal peptide (VIP). Interestingly, systemic administration of VIP reversed the inflammation-induced disruption of the blood-CSF barrier integrity. In conclusion, this study shows for the first time that *H. suis* infection induces inflammation in the brain and that the choroid plexus is a novel player in the stomach-brain axis.

Introduction

The healthy human gastrointestinal tract harbors approximately 100 trillion commensal bacteria (Clemente et al., 2012; Foster and McVey Neufeld, 2013). An increasing number of recent studies indicate that the shifts in the gastrointestinal microbiome caused by a broad variety of triggers, including gastrointestinal infections, might have a significant effect on our health (Ochoa-Reparaz and Kasper, 2014; Walters et al., 2014; Zhang et al., 2015). Indeed, dysbiosis has been associated with a wide range of human health problems (Clemente et al., 2012; Zhang et al., 2015; Boulange et al., 2016), including neurological disorders such as depression, anxiety-like behavior, autism, Alzheimer's disease and Parkinson's disease (Parracho et al., 2005; Bercik et al., 2011; Bravo et al., 2011; Zhang et al., 2015; Hu et al., 2016; Mulak and Bonaz, 2015).

The genus *Helicobacter* is a group of gram-negative, spiral-shaped bacteria colonizing the gastrointestinal tract of humans and animals. *Helicobacter pylori* (*H. pylori*) is the best-known gastric human pathogen and is one of the major causes of gastric cancer (Kusters et al., 2006). Besides *H. pylori*, other *Helicobacter* species, referred to as gastric non-*Helicobacter pylori Helicobacter* (NHPH) species, have also been associated with severe human gastric malignancies (Haesebrouck et al., 2009). The most common NHPH species in humans is *H. suis* (Haesebrouck et al., 2009), which naturally colonizes the stomach of pigs (Liang et al., 2013) and non-human primates (Bosschem et al., 2016). Most likely, transmission of *H. suis* occurs through direct or indirect contact between animals and humans. Additionally, *H. suis* can be present and survive in minced pork, suggesting that raw or undercooked pork may be a source of *H. suis* infection of humans (De Cooman et al., 2013).

Recently, an exceptionally high frequency (27%) of *H. suis* DNA was found in gastric biopsies from human patients with Parkinson's disease compared to a control group with no clinical parkinsonism (2%) (Blaecher et al., 2013). Besides, *H. suis* DNA was also detected in a blood sample from a patient with both parkinsonism and Alzheimer's disease (unpublished results). The patient was a middle-aged male, who was treated with tetracycline, clarithromycin, bismuth and a proton pump inhibitor to eradicate *H. suis.* This therapy resulted in a temporary improvement of the well-being as well as the gastric and neurological symptoms of the patient (Dobbs et al., 2013). This finding suggests that the inflammatory effects caused by *Helicobacter* are not restricted to the stomach.

To prevent disturbance of brain homeostasis due to alterations in the systemic blood composition, the brain is protected by a series of barriers, including the blood–brain barrier (BBB) and the blood–cerebrospinal fluid (CSF) barrier (De Bock et al., 2014). It has been suggested that systemic inflammation may contribute to the impairment of the BBB (Kortekaas et al., 2005; Stolp and Dziegielewska, 2009) and the blood–CSF barrier (Vandenbroucke et al., 2012; Demeestere et al., 2015). Furthermore, several neurodegenerative disorders, including Parkinson's disease and Alzheimer's disease, have been associated with disruption of the BBB (Stolp and Dziegielewska, 2009; Lee and Pienaar, 2014; Li et al., 2015) and the blood–CSF barrier (Vawter et al., 1996; Pisani et al., 2012; Bergen et al., 2015; Balusu et al., 2016a; Gorle et al., 2016). Because it has been proposed that *Helicobacter* infections cause systemic inflammation (Blaecher et al., 2013; Alvarez-Arellano and Maldonado-Bernal, 2014), gastric infection with *H. suis* could influence the integrity of the brain barriers and thereby contribute to the pathophysiology of neurodegenerative disorders.

In this study, we investigated the effect of gastric *H. suis* infection on brain homeostasis. Our results show that *H. suis* induces inflammation not only in the stomach, but also in the brain. Moreover, we identified two possible pathways of communication between the gastrointestinal tract and the brain. On the one hand, leakage of the gastrointestinal barrier induced systemic inflammation and caused loss of the blood-CSF barrier integrity. On the other hand, *H.* suis-induced alterations in VIP signaling might affect brain homeostasis because we found that administration of VIP can reverse systemic inflammation-induced blood-CSF barrier leakage and brain inflammation.

Materials and methods

Mice. For the *Helicobacter* infection experiments, female C57BL/6 mice (5 weeks old) were purchased from Harlan (Harlan Laboratories, Horst, The Netherlands). The animals were housed in filtertop cages in groups of 5-10 mice/cage with free access to food and water and with a 12-hour light/12-hour dark cycle in a conventional animal house. All experiments were approved by the ethical committee of the Faculty of Veterinary Medicine of Ghent University. For the VIP experiment, female C57BL/6 mice (7 weeks old) were purchased from Janvier (Janvier Labs, Le Genest-Saint-Isle, France). Mice were housed with 4-6 mice/cage in a specific pathogen-free animal facility with free access to food and water and with a 14-hour light/10-hour dark cycle. This experiment was approved by the ethics committee of the Faculty of Sciences of Ghent University.

Cultivation of *H. suis* bacteria. *H. suis* bacteria (strain HS1) were grown biphasically on 1.5% *Brucella* agar (Immunosource, Halle-Zoersel, Belgium), supplemented with 1 ml skirrow (Oxoid, Hampshire, United Kingdom), 3 ml vitox (Oxoid, United Kingdom), 5 µg/ml amphotericin B (Sigma-Aldrich, Germany), 0.05% HCL (Sigma-Aldrich, Taufkirchen, Germany) and 20% inactivated fetal bovine serum (Perbio, Thermo Scientific Hyclone, Cramlington, United Kingdom) with on top *Brucella* broth (Immunosource, Belgium), supplemented with 0.05% HCL (Sigma-Aldrich, Germany)(Baele et al., 2008). The plates were incubated at 37°C under micro-aerobic conditions (10% CO_2, 5% O_2 and 85% N_2). Using a counting chamber (Neubauwer Improved Assistant) the bacteria were counted and diluted to 10^8 viable *H. suis* bacteria/ml.

***In vivo* infection procedure.** At the age of 6 weeks, animals were anaesthetized with 2.5% isoflurane and intragastrically inoculated with either 300 µl of a stock solution containing 10^8 viable *H. suis* bacteria/ml or with 300 µl of the growth medium of the bacteria (control group). One month after infection, mice were euthanized for further analysis by the administration of an overdose of ketamine/xylazine followed by decapitation. In order to perform the different analyses, mice were divided into 4 different groups: the first group of mice (10 controls and 10 *H. suis* infected mice) was used to determine blood-CSF barrier and BBB permeability, the second group (5 controls and 5 *H. suis* infected mice) was used for quantification of gastrointestinal permeability, the third (5 controls and 5*H. suis* infected mice) and fourth group (10 controls and 10 *H. suis* infected mice) were used for immunohistochemical stainings and DNA, RNA and protein analyses of stomach and brain samples, respectively.

VIP experiment. Mice were intraperitoneally (ip) injected with lipopolysaccharide (LPS) from *Salmonella enterica* ser. Abortus equi (Sigma) dissolved in D-PBS. The dose was 120 µg/20 g body weight. Control animals received ip injections of D-PBS. Additionally, mice were injected twice with 5 nmol VIP (05-23-2101-1MG,

Millipore, Merck, Overijse, Belgium) intravenously (iv): together with and 2h after LPS injection. Six hours after LPS injection, CSF was isolated and used to determine blood-CSF permeability as described below and choroid plexus and hippocampus were isolated for gene expression analysis.

Tissue collection. For RNA and protein analysis, mice were transcardially perfused with D-PBS/heparin (0.2% heparin) supplemented with 0.5% bromophenol blue. The stomach (including a small part of the duodenum) was collected and cut open via the curvatura major. After rinsing with sterile Hank's Buffered Salt Solution (Gibco by Thermo Fisher Scientific, Gaithersburg, USA), samples from the antrum and the corpus of the stomach and duodenum were collected and stored in RNA*later* (Qiagen, Antwerpen, Belgium) at -70°C or snap frozen in liquid nitrogen for RNA and protein extraction, respectively. Brain tissue was dissected out. The choroid plexus from all four ventricles was collected and snap frozen in liquid nitrogen, while hippocampus samples were stored in RNA*later* (Qiagen).

For immunohistochemical analyses, mice were transcardially perfused with 4% paraformaldehyde (PFA). Brain tissue and samples from the antrum and corpus of the stomach and duodenum were collected. The brain was divided into the left and right hemisphere. The right hemisphere was successively fixated in 4% PFA and embedded in paraffin for paraffin sections. The stomach and duodenum samples were either embedded in cryoprotectant (Thermo Scientific™, Waltham, USA), underwent rapid freezing on dry ice and were stored at -70°C or were fixated overnight in a solution of 3.7% formaldehyde (Chem-lab, Zedelgem, Belgium) and subsequently embedded in paraffin (Labo-nord, VWR, Leuven, Belgium).

DNA extraction and quantification of colonizing *H. suis* bacteria in the stomach and duodenum. Stomach and duodenum samples stored in RNA*later* were homogenized using the MagNAlyser (Roche diagnostics, Vilvoorde, Belgium) and RNA and DNA were separated as described before (Joosten et al., 2013). The number of colonizing *H. suis* bacteria per mg tissue was determined in the DNA samples using Real-Time (RT)-quantitative (q) Polymerase Chain Reaction (PCR). RT-qPCR detection of *H. suis* DNA was performed using the *ureA*-based primers (**Table 1**) (Blaecher et al., 2013) and the *s*tandard was generated as described before (O'Rourke et al., 2004). Standard and samples were run in duplicate on a CFX384™ RT-qPCR System with a C1000 Thermal Cycler (Bio-Rad, Temse, Belgium) according to the manufacturer's instructions.

RNA extraction and RT-qPCR for gene expression. Using the RNeasy mini kit (Qiagen), total RNA was extracted from the antrum and the corpus of the stomach, the duodenum, hippocampus and choroid plexus according to the manufacturer's instructions and measured using Nanodrop equipment (Nanodrop ND-1000, Fisher Scientifics). cDNA was synthesized using a cDNA Synthesis Kit (Bio-Rad). RT-qPCR analysis of the stomach and duodenum samples was performed on a CFX384^{TM} RT-qPCR System with a C1000 Thermal Cycler (Bio-Rad), using the 2xSensimix (Sybr No-Rox kit; Bioline, London, UK), while for brain samples the RT-qPCR was performed on the Light Cycler 480 system (Roche) using the 2xSensimix (Sybr No-Rox kit; Bioline). Expression levels were normalized to the expression of 3 reference genes: *Hprt, H2afz* and *Ppia* for stomach and duodenum samples, *Hprt*¸ *Rpl* and *Ubc* for choroid plexus samples and *Gapdh*, *Rpl* and *Ubc* for hippocampus samples. The primer sequences are shown in **Table 1**.

Cytokine/chemokine measurements. The snap frozen gastrointestinal samples were lysed using lysis buffer containing 0.5% CHAPS (Sigma-Aldrich) and a protease inhibitor complete tablet (Roche Applied Science; 11

873 580 001). Protein concentration of the samples was measured using the Pierce BCA protein assay kit (Thermofisher Scientific). Cytokines and chemokines in serum, CSF and protein lysates were measured using the Bio-Plex cytokine assays (Bio-Rad) according to the manufacturer's instructions.

Quantification of the gastrointestinal permeability. Gastrointestinal permeability was determined as previously described (Vandenbroucke et al., 2014; Van Hauwermeiren et al., 2015). Briefly, 4 kDa FITC-dextran (Sigma) was intragastrically administered five hours before collection of blood. Blood was collected in EDTA tubes (Sarstedt, Wexford, Ireland) by means of heart punction followed by centrifugation. Subsequently, plasma was isolated and diluted twice in sterile D-PBS. Gastrointestinal leakage was determined by measurement of fluorescence at $\lambda_{ex}/\lambda_{em}$ = 488/520 nm.

Quantification of blood-CSF barrier and BBB permeability. Blood-CSF barrier and BBB permeability were determined as previously described (Vandenbroucke et al., 2012; Brkic et al., 2015). Briefly, 4 kDa FITC-dextran (Sigma) was injected iv 1 h before CSF collection. CSF was obtained from the fourth ventricle using the cisterna magna puncture method (Vandenbroucke et al., 2012; Brkic et al., 2015). Subsequently, mice were perfused with D-PBS/heparin (0.2% heparin) and brain tissue was isolated. CSF samples were diluted 100-fold in sterile D-PBS, and blood-CSF barrier leakage was determined by measurement of fluorescence at $\lambda_{ex}/\lambda_{em}$ = 488/520 nm. Brain samples were cut into small pieces, incubated overnight at 37°C in formamide while shaking and supernatant was collected after centrifugation for 15 min at maximum speed. Brain fluid was diluted 2-fold in sterile D-PBS, and BBB leakage was determined by measurement of fluorescence at $\lambda_{ex}/\lambda_{em}$ = 488/520 nm.

Histopathology and immunohistochemistry. In order to evaluate inflammation in the stomach and duodenum, paraffin sections of 5 µm were cut using a microtome (Microm, Prosan, Thermo-Fisher). Sections were stained with Hematoxylin Gill III Prosan (Merck) and Eosine Yellow (VWR) according to standardized protocols. Gastric tissue was examined using a light microscope (Leica DM2000). The H&E stainings of the stomach were used to determine the grade of overall gastritis, using the modified visual analog scale similar to the adapted Updated Sydney System as previously described (Flahou et al., 2010).

For zonula occludens 1 (ZO1) immunostaining of the stomach, 30 µm cryosections were cut using a cryostat (Micron HM500) and mounted on slides. After air drying for 1 h, sections were fixated with 1% PFA for 10 min, washed for 5 min in PBS, and permeabilized for 10 min with 0.1% NP-40. After two rinses with PBS, samples were blocked for 1 h at room temperature with 5% goat serum and then incubated with primary antibody (Invitrogen, ZO1, 1:100, Merelbeke, Belgium). After incubation overnight at 4°C, they were washed with PBS-T and incubated with secondary antibody (Thermo Scientific; goat anti-rabbit-DyLight633, 1:300) diluted in 5% serum for 90 min at room temperature. Finally, a Hoechst staining was performed to counterstain the nuclei, the slides were dehydrated and xylene-based mounting medium was applied. Cells were visualized using a confocal microscope (Zeiss LSM780).

For the MUC13 staining of the stomach sections of 5 µm were cut from paraffin embedded tissue, using a microtome (Microm, Prosan, Thermo-Fisher), and placed on a microscope slide. Deparaffinization was performed by the addition of successively, xylene for 10 min, isopropyl alcohol for 5 min, 95% ethanol for 5 min, 50% ethanol for 5 min and aqua destillata for 5 min. Antigen retrieval was performed in citrate buffer (10 mM, pH6) using a pressure cooker, where after samples were rinsed with washing buffer (Dako, Santa-Clara,

USA). Endogenous peroxidase was blocked for 5 min with peroxidase blocking reagent (Dako) and rinsed with aqua destillata and washing buffer (Dako). Successively slides were incubated for 30 min with the primary in-house polyclonal mouse MUC13 antibody, diluted in antibody diluent (1/1000, Dako, S3022). After a rinse step with washing buffer (Dako), samples were incubated with labeled polymer-HRP anti-mouse for 30 min at room temperature (Evision+ system-HRP (DAB) (K4007)). Samples were rinsed 2 times with washing buffer (Dako), where after DAB + substrate (Dako) and DAB + chromogen (Dako) were added during 5 min. Finally, a counterstaining with hematoxyllin was performed during 10 min, followed by a rinse step of 1 min in running tap water and a dehydration step. Gastric tissue was visualized using a light microscope (Leica DM2000).

For IBA1 immunostaining, paraffin sections of the brains were processed as described before using the anti-IBA1 antibody (Wako, 019-19741, Neuss, Germany) (Brkic et al., 2015). Imaging of whole brain sections was done using Zeiss Axio Scan.Z1 followed by quantification using Fiji (http://fiji.sc/Fiji). Quantification of the percentage of brown color was done via color thresholding, with correction for the total amount of tissue, while microglia were classified into resting and activated in the subventricular zone according to adopted criteria (Hains and Waxman, 2006). Representative images were taken using an Olympus BX51 microscope.

For Claudin 1 (CLDN1) (Invitrogen, 51-9000) and VIP (Immunostar, 20077, Hudson, USA) immunostaining, paraffin-embedded brain and stomach tissues were sectioned at 5 µm, dewaxed, and rinsed in water and PBS. Antigen retrieval was done using citrate buffer (Dako; S2031), followed by washing in PBS. For CLDN1 staining, endogenous peroxidase was blocked by incubating the slides in 3% H_2O_2 in methanol for 10 min, followed by washing in PBS. All samples were blocked with 5% goat serum in antibody diluent (Dako; S2022) for 30 min at RT, followed by incubation overnight at 4°C with primary antibody (CLDN1, Invitrogen, 51-9000, 1:100; VIP, Immunostar, 1/50). The next day, slides were washed with PBS. For CLDN1 immunostaining, slides were incubated with secondary antibody coupled to HRP (Dako; E-K4003), and washed with PBS. An amplification step was performed using tyramide (TSA kit, Perkin Elmer, Zaventem, Belgium) and visualization was done using ABC (Vector, Brussel, Belgium) and DAB. Slides were counterstained with haematoxylin, dehydrated and xylene-based mounting media was applied. Cells were visualized using an Olympus BX51 microscope. For VIP immunostaining, slides were incubated with secondary antibody (Thermo Scientific; goat anti-rabbit-DyLight633, 1:1000) diluted in 5% serum for 1 h at room temperature. Finally, a Hoechst staining was performed to counterstain the nuclei, the slides were dehydrated and xylene based mounting medium was applied. Cells were visualized using a confocal microscope (Leica TCS SP5).

Statistics. Data were analyzed by Mann-Whitney test using GraphPad Prism or SPSS and are presented as means $\pm$ standard error of mean (SEM), unless stated otherwise. Significance levels are indicated on the graphs *, $0.01 \leq P < 0.05$; **, $0.001 \leq P < 0.01$; ***, $0.0001 \leq P < 0.001$; ****, $P < 0.0001$.

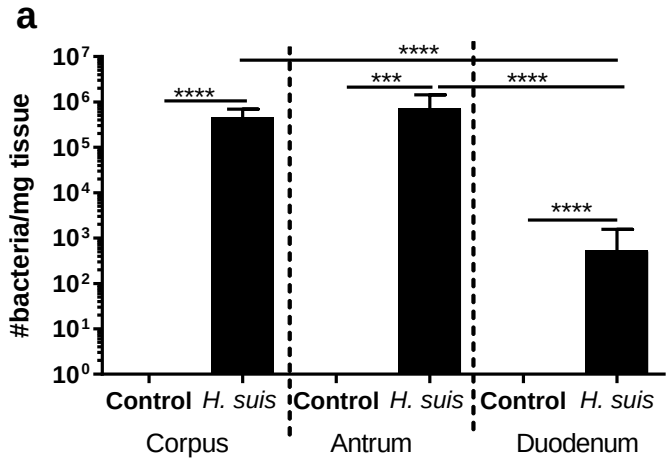
Results

Colonization *by H. suis* induces gastric inflammation

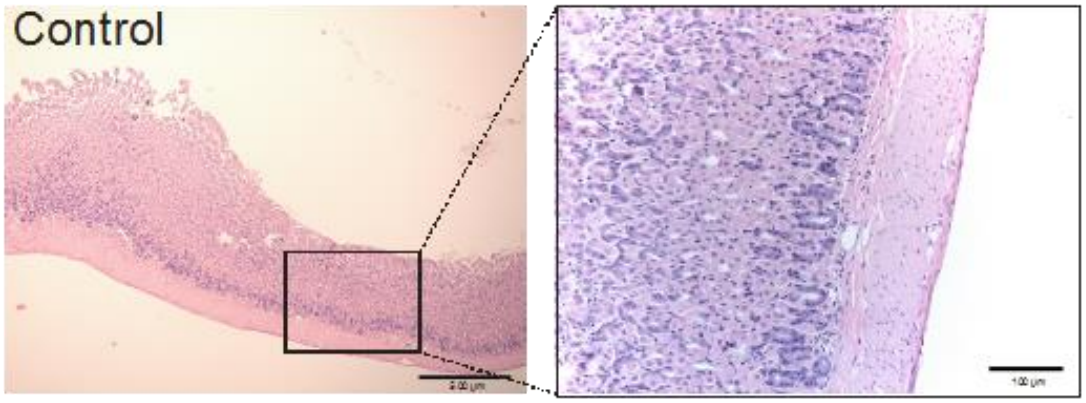
One month after infection, colonization by *H. suis* was confirmed by analyzing the stomach and small intestine. The mouse stomach consists of a non-glandular part and a glandular part. In this study, we analyzed only the glandular part, consisting mainly of the corpus and the antrum of the stomach. As shown in Figure 1a, the corpus and antrum of the stomach of *H. suis*-infected animals were colonized by an average of 4.51E+05 and 7.30E+05 bacteria per mg tissue, respectively. No *H. suis* DNA was detected in the stomach of control mice (Figure 1a). Additionally, *H. suis* DNA was also detected in the duodenum of infected mice but at a lower level than in the stomach (Figure 1a).

Analysis of H&E-stained stomach of control animals showed no or negligible infiltration of mononuclear and polymorphonuclear cells in the mucosa and the submucosa, which agrees with the normal histological image of the stomach (Figure 1b). In contrast, pronounced infiltration of mononuclear and polymorphonuclear cells was detected in the stomach of *H. suis*-infected animals (Figure 1c), indicating gastric inflammation as scored by the Updated Sydney System (Figure 1d). Furthermore, inflammation was also associated with changes in the expression levels of pro-inflammatory cytokines. Gene expression analyses revealed that the mRNA levels of the murine interleukin 8 (*IL8*) homologue keratinocyte chemoattractant (*Kc*) and lipopolysaccharide-induced CXC chemokine (*Lix*) were significantly up-regulated in corpus (Figure 1e) and in antrum (Figure 1f) of *H. suis*-infected mice. A significant increase in gene expression of *Il1β* was observed only in the corpus of those animals (Figure 1e) and a significant decrease of *Il10* only in the antrum (Figure 1f). Additionally, a significant increase in protein levels of IL1β and IL17 was observed in the corpus of the stomach of *H. suis*-infected mice (Figure 1h-i). In contrast, no significant changes in the expression of cytokines and chemokines were found in the duodenum of mice infected with *H. suis* (Figure 1g).

Figure 1



b



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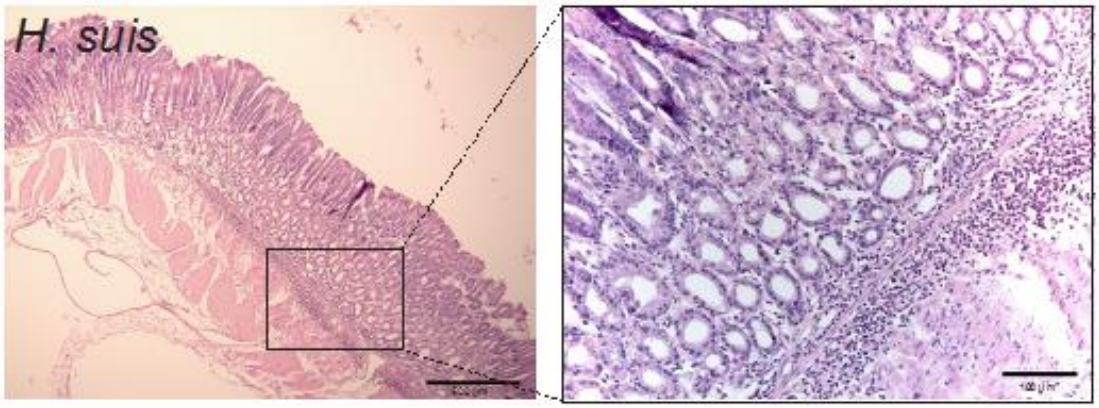


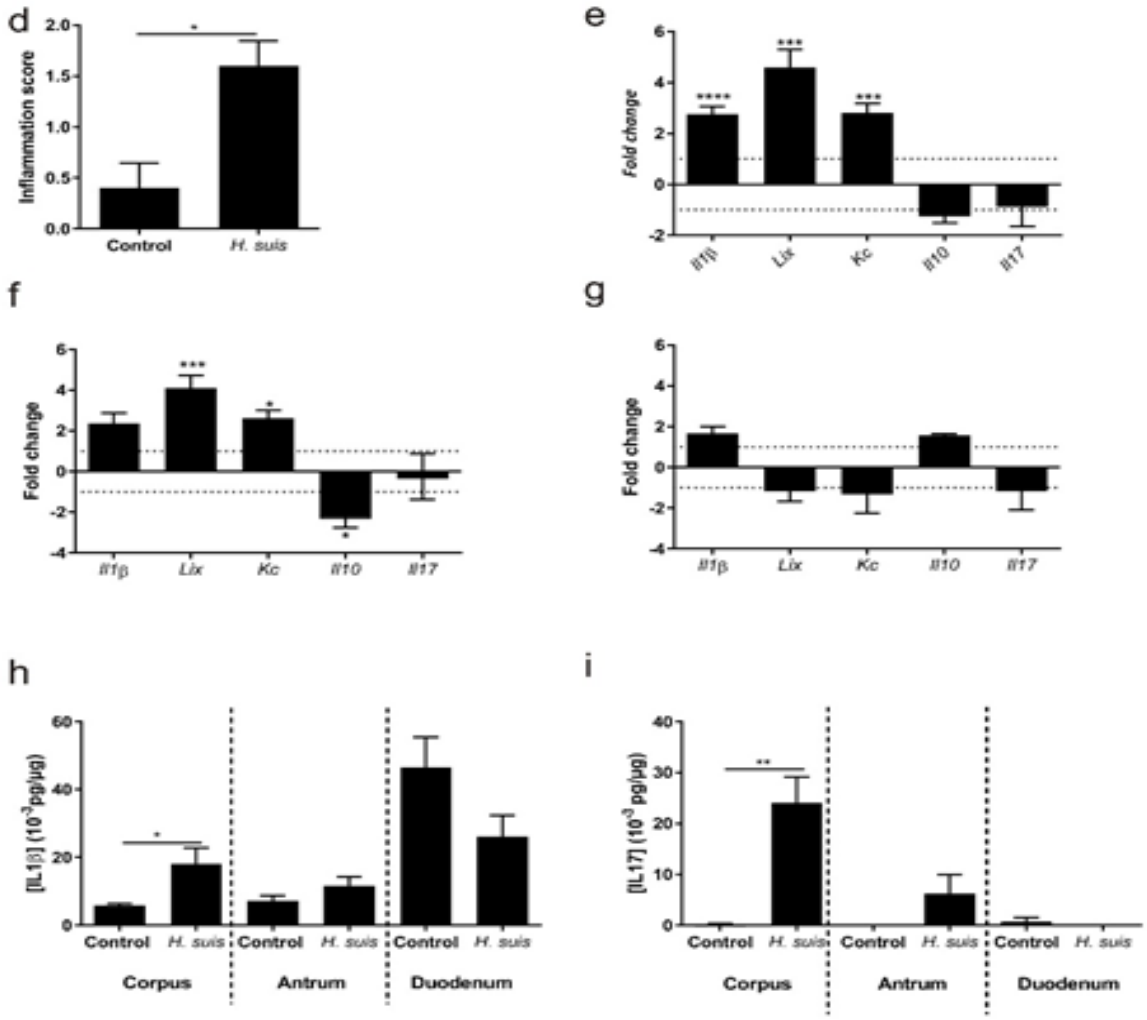


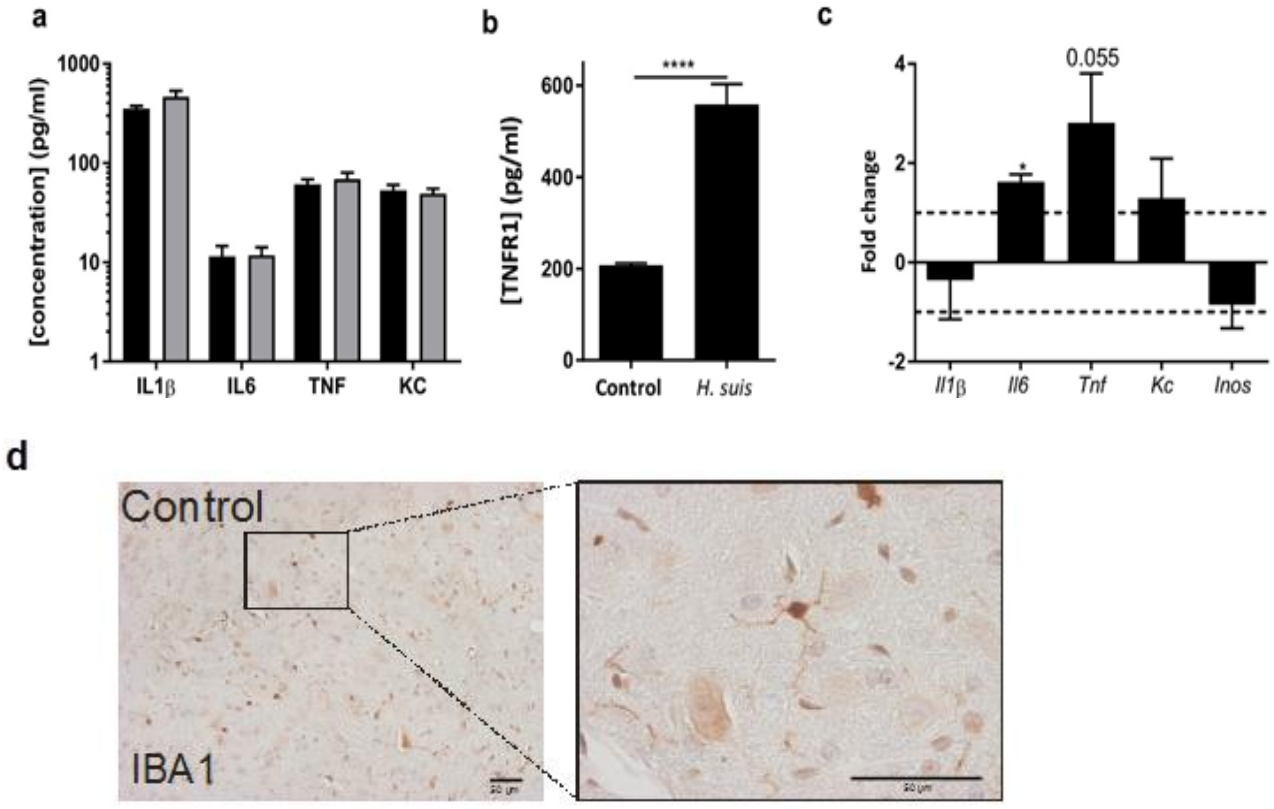
Figure 1. Analysis of colonization and inflammation in the stomach and duodenum after one month of *Helicobacter suis* (*H. suis*) infection. (a) *H. suis* colonization in stomach (corpus and antrum) and duodenum of C57BL/6 mice one month after *H. suis* infection compared to control mice (n = 7-10). **(b-c)** Representative H&E images of stomach sections of control (b) and *H.* suis-infected (c) C57BL/6 mice one month after infection. **(d)** Inflammation score based on H&E staining of stomach of *H. suis*-infected and control mice (n = 5). **(e)** Inflammatory gene expression analysis (represented as fold change) of interleukin 1β (*Il1β*), *lipopolysaccharide-induced CXC chemokine* (*Lix*), keratinocyte chemoattractant (*Kc*), *Il10* and *Il17* in corpus of stomach in C57BL/6 mice after one month of infection with *H. suis* compared to control mice (n = 7-10). **(f-g)** Expression of the inflammatory genes interleukin 1β (*Il1β*), lipopolysaccharide-induced CXC chemokine (*Lix*), keratinocyte chemoattractant (*Kc*), *Il10* and *Il17* in the antrum of the stomach (f) and in the duodenum (g) in C57BL/6 mice after one month of infection with *Helicobacter suis* (*H. suis*) compared to control mice, represented as fold change (n = 3-9). **(h-i)** Levels of IL1β (h) and IL17A (i) in protein lysates determined using Bioplex of stomach (corpus and antrum) and duodenum after inoculation with *H. suis* or growth medium. Data were analyzed by Student's t-test (n = 5).

Gastric *H. suis* infection induces inflammation and activation of microglia in the brain

To determine whether *H. suis* affects the brain, cerebrospinal fluid (CSF) and brain tissue were analyzed for inflammation. Bioplex analysis of CSF revealed no significant changes in cytokines and chemokines (Figure 2a).

However, a significantly higher concentration of soluble tumor necrosis factor receptor 1 (sTNFR1) was found in the CSF of *H. suis*-infected mice (Figure 2b). Despite the limited changes in the CSF, a significantly stronger expression of *Il6* was detected in the brain hippocampus, while *Tnf, Il1β*, *Kc* and inducible nitric oxide synthase (*iNos*) expression was unaffected (Figure 2c). Since microglia are important mediators of brain inflammation, we stained brain sections for ionized calcium binding adaptor molecule 1 (IBA1), a marker for microglia (Figure 2d-e). Automatic analysis showed a significant increase in the total IBA1-positive signal in the brain (Figure 2f). Additionally, manual quantification of the percentage of activated microglia in the subventricular zone, based on the morphology of IBA1-positive cells, revealed increased numbers of ramified microglia in *H. suis*-infected animals compared to control mice (Figure 2g). These data indicate that *H. suis* activates a pro-inflammatory response in the brain.

Figure 2



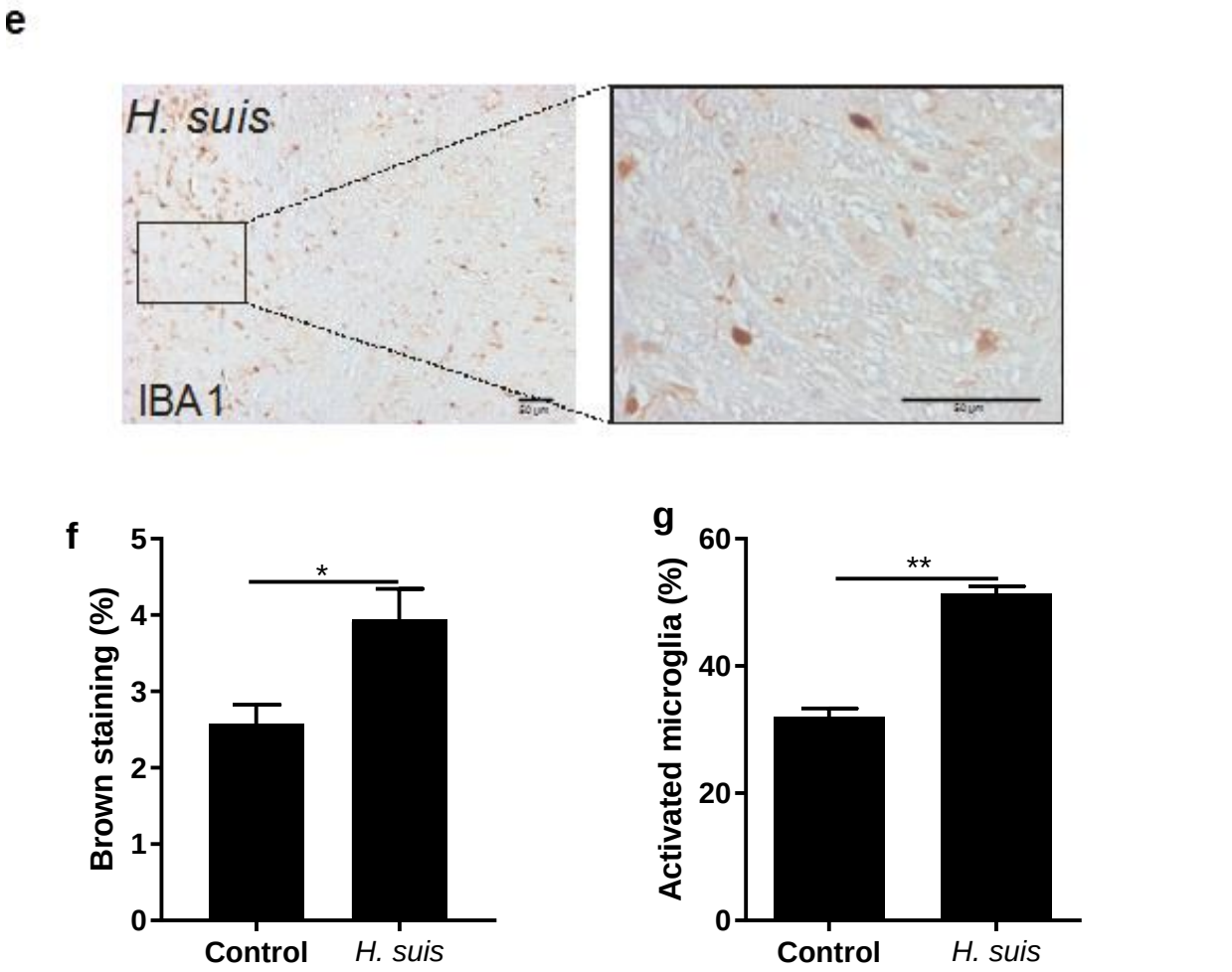


Figure 2. Analysis of brain inflammation in response to one month *Helicobacter suis* (*H. suis*) infection. (a) Levels of interleukin 1β (IL1β), IL6, tumor necrosis factor (TNF) and keratinocyte chemoattractant (KC) in cerebrospinal fluid (CSF) determined using Bioplex. Data were analyzed by Student's T-test (n = 4-5). **(b)** Concentration of TNFR1 in the cerebrospinal fluid (CSF) of control mice and mice infected with *H. suis* (n = 10). **(c)** Expression of inflammatory genes *Il1β*, *Il6*, *Tnf*, *Kc* and inducible nitric oxide synthase (iNos) in the hippocampus of C57BL/6 mice infected with *H. suis* compared to control mice, represented as fold change. Data were analyzed by Student's T-test (n = 3-5). **(d-e)** Representative images of ionized calcium-binding adapter molecule 1 (IBA1) staining of brain sections at the subventricular zone from C57BL/6 of control mice (d) and of mice after one month infection with *H. suis* (e). **(f)** Quantification of the percentage brown staining (IBA1) in the whole brain of *H. suis*-infected and negative control mice (n = 5). **(g)** Quantification of the percentage activated microglia in *H. suis*-infected and negative control mice (n = 5).

***H. suis* infection induces loss of the blood-CSF barrier but does not affect BBB integrity.**

The presence of tight brain barriers, including the BBB and the blood-CSF barrier, ensure brain homeostasis (De Bock et al., 2014), but several inflammatory triggers are known to disrupt barrier integrity (Nielsen et al., 2012; Vandenbroucke et al., 2012; Alvarez-Arellano and Maldonado-Bernal, 2014; Brkic et al., 2015; Gorle et al., 2016). Here, we studied whether the effect of *H. suis* on the brain is associated with loss of barrier integrity. No change in BBB permeability was observed (Figure 3a). In contrast to the BBB, a significant increase in blood–CSF barrier permeability was seen after one month of infection (Figure 3b). Gene expression analysis of the blood–CSF barrier located at the choroid plexus revealed that several tight-junction genes were affected by *H. suis*

infection. As shown in Figure 3c, gene expression of claudin 1 (*Cldn1*) and occludin (*Ocln*) was significantly down-regulated in the presence of *H. suis* infection. Other tight-junction genes, such as *Cldn5,* zona occludens 1 (*Zo1*) and *Zo3* decreased, but not significantly so. Finally, immunostaining was performed to determine the subcellular localization of CLDN1. The arrowheads in Figure 3d-e point to loss of apical localization of CLDN1 in the choroid plexus epithelial cells of *H. suis*-infected mice. These data indicate that *H. suis* infection compromises the functionality of the blood–CSF barrier but does not affect the integrity of the BBB.

Figure 3

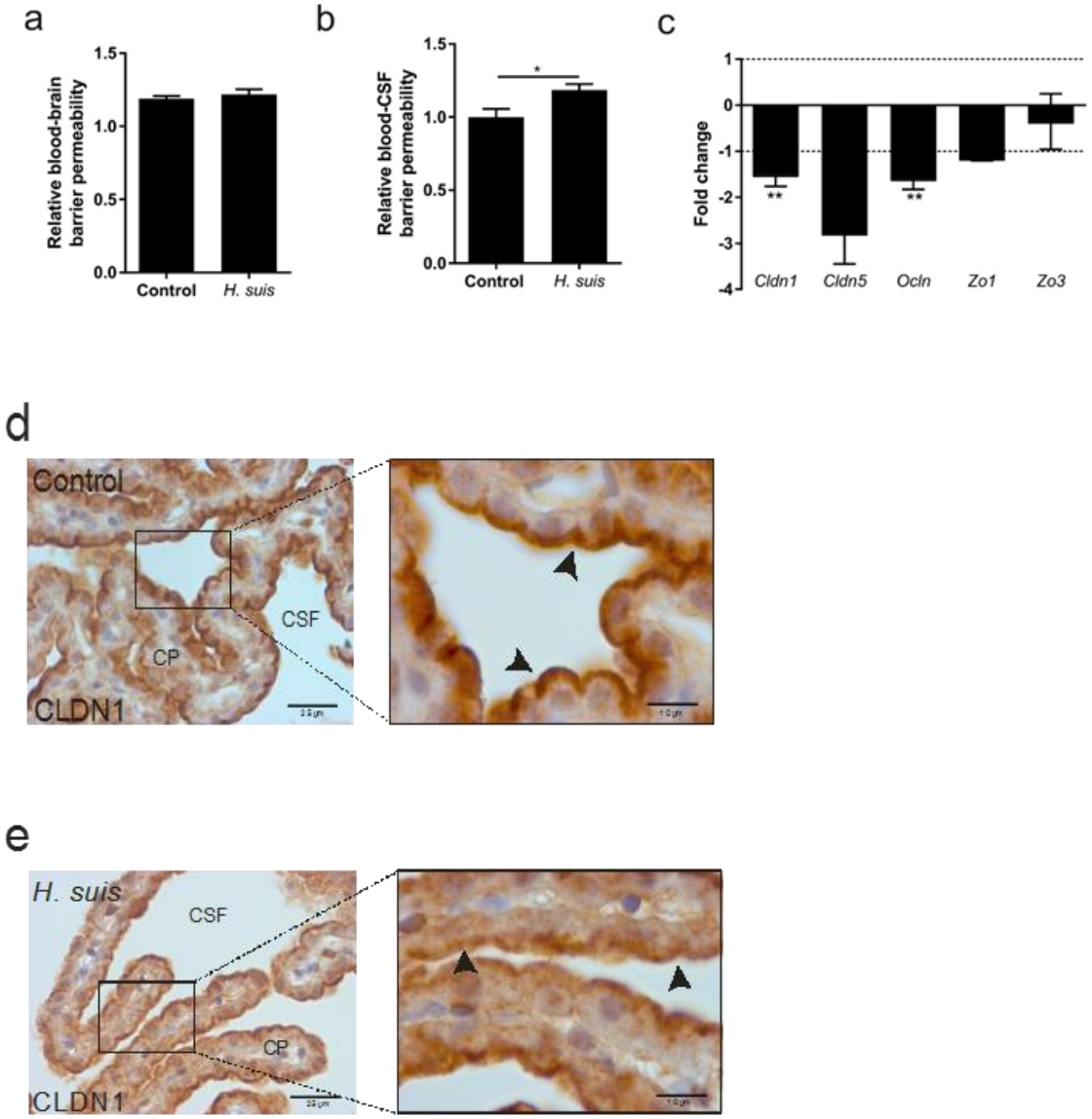


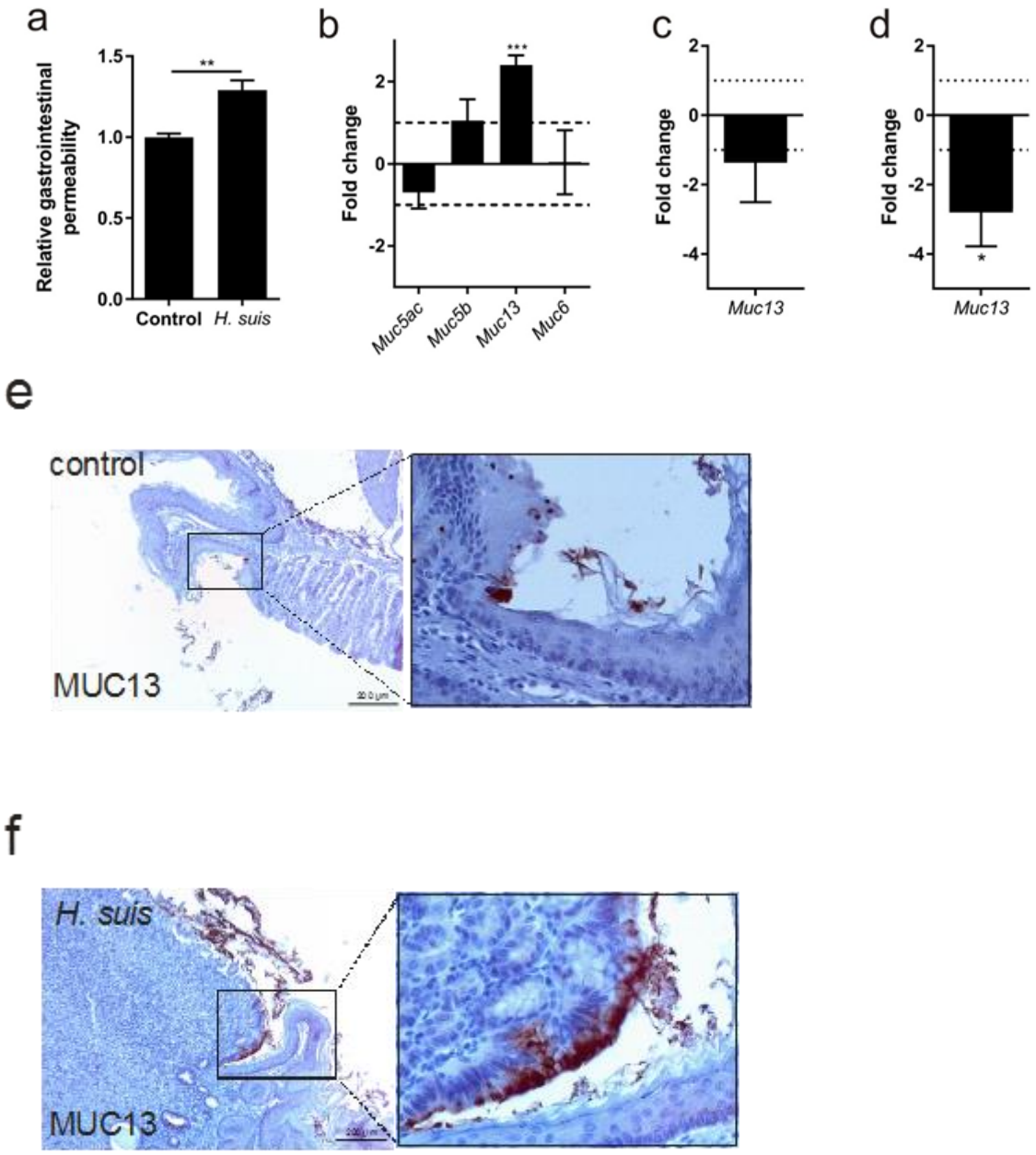
Figure 3. Brain barrier analysis in response to one month of *Helicobacter suis* (*H. suis)* infection. (a) Relative blood-brain barrier (BBB) permeability one month after inoculation with growth medium or *H. suis* (n = 5). **(b)** Relative blood-cerebrospinal fluid (CSF) barrier permeability one month after inoculation with growth medium or *H. suis* (n = 8–10). **(c)** Expression of the tight junction genes claudin 1 (*Cldn1*), *Cldn5*, occludin (*Ocln*), zonula occludens (*Zo1*) and *Zo3* in choroid plexus tissue from C57BL/6 mice infected with *H. suis* compared to control mice (n = 5). **(d-e)** Representative images of choroid plexus tissue stained for CLDN1 of control mice (d) and *H. suis*-infected mice (e). CP: choroid plexus.

H. suis infection induces loss of gastric mucosal barrier integrity and subsequent systemic inflammation

Increased gastrointestinal permeability, which could lead to leakage of pro-inflammatory mediators and bacterial metabolites from the gut into the systemic circulation, is considered one of the gut–brain axis pathways (Cryan and Dinan, 2012; Mayer et al., 2014). In our study, we used oral gavage of FITC-dextran to investigate the influence of gastric *H. suis* infection on the gastrointestinal barrier. Plasma fluorescence analysis revealed a significant increase in gastrointestinal permeability after one month of *H. suis* infection (Figure 4a). The gastric mucosal barrier is composed of the gastric mucosa (epithelium, lamina propria and muscularis mucosa), with a mucus layer on top (Linden et al., 2002; Kawakubo et al., 2004; Yamashiro, 2006, McGuckin, 2011 #321). This gastric mucus layer, consisting mainly of secreted mucins, is the first barrier pathogens encounter. The membrane-associated mucins, located underneath this mucus layer, also provide a barrier, thereby limiting access to the epithelial cell surface (McGuckin, 2011). Changes in mucin distribution and expression might affect the integrity of the gastric mucosal barrier (Kufe, 2009). Therefore, we analyzed gastric mucin expression. RT-qPCR analysis revealed a significantly increased gene expression of the transmembrane mucin 13 (*Muc13*) in the corpus of *H. suis*-infected mice, while the expression of *Muc5ac, Muc5b* and *Muc6* were unaffected (Figure 4b). This finding was confirmed histologically (Figure 4e-f): compared to control mice (Figure 4e), apical membrane and cytoplasmatic MUC13 staining (brown) was much more pronounced in the mucus-secreting epithelial cells of the stomach of *H. suis*-infected mice (Figure 4f). In contrast, *Muc13* gene expression did not change in antrum and was significantly down-regulated in duodenum of *H. suis*-infected animals (Figure 4c-d).

The gastric epithelium located underneath the mucus layer is formed by epithelial cells connected by tight junction proteins (Caron et al., 2015). We performed immunostaining to determine whether the *H. suis*-induced alterations in gastrointestinal barrier integrity were correlated with changes in junctional proteins in the stomach. Confocal imaging revealed that expression of ZO1 was disturbed. Compared to control mice, in which ZO1 was enriched mainly at the apical side of the stomach epithelial cells (Figure 4g), the ZO1 signal was much more diffuse in stomach samples from *H. suis*-infected animals (Figure 4h). The changes in the expression of both MUC13 and ZO-1 might thus explain the increased gastrointestinal leakage in the presence of *H. suis* infection.

Figure 4


a
Relative gastrointestinal permeability
1.5
1.0
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Control
H. suis
b
Fold change
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Muc5b
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c
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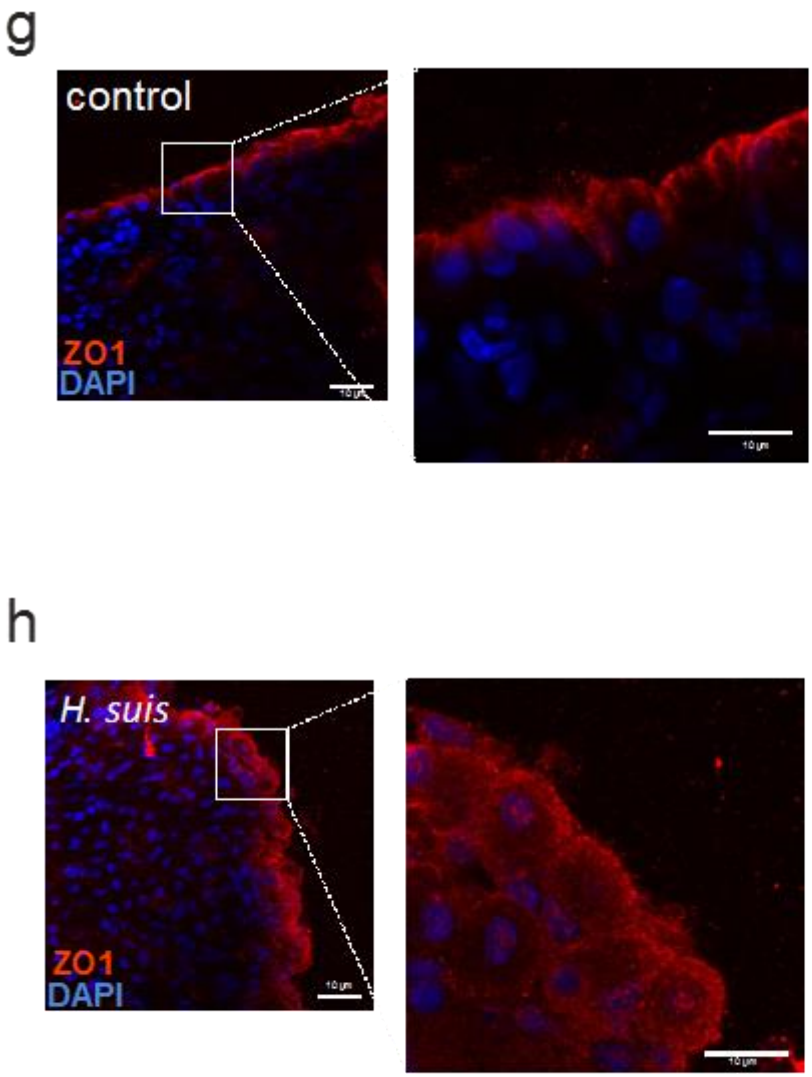


Figure 4. Analysis of the gastrointestinal barrier after one month of *Helicobacter suis* (*H. suis*) infection. (a) Relative gastrointestinal permeability of control mice compared to mice infected with *H. suis* (n = 5). **(b)** Expression of mucins *Muc5ac*, *Muc5b*, *Muc13* and *Muc6* in the corpus of the stomach of C57BL/6 mice inoculated with *H. suis* and of control mice, represented as fold change (n = 7-10). **(c-d)** Expression of the mucin *Muc13* in antrum of the stomach (c) and in duodenum (d) in C57BL/6 mice one month after infection with *Helicobacter suis* (*H. suis*) compared to control mice, represented as fold change (n = 5-9). **(e-f)** Representative images of immunostaining of MUC13 in the stomach of control mice (e) and *H. suis* infected mice (f). **(g-h)** Representative confocal images of zonula occludens 1 (ZO1) staining in the stomach of control mice (g) and *H. suis*-infected C57BL/6 mice (h).

Disruption of the gastric mucosal barrier might result in leakage of pro-inflammatory cytokines, chemokines and bacterial metabolites from the stomach into the blood, leading to systemic inflammation (Cryan and Dinan, 2012). Additionally, recent studies indicated that alterations in circulating cytokine levels may directly influence the functionality of the brain barriers and the brain (Cryan and Dinan, 2012; Vandenbroucke et al., 2012; Balusu et al., 2016b). Therefore, the presence of systemic inflammation was evaluated by analyzing the serum of

control and *H. suis*-infected mice. As shown in Figure 5a, the levels of the pro-inflammatory cytokine IL1β and chemokine MIP1α increased significantly in the serum of *H. suis*-infected mice. These findings indicate that the loss of blood-CSF barrier integrity observed in our study might have been caused by a low grade, systemic inflammation induced by *H. suis*. In agreement with this finding, gene expression analysis of choroid plexus tissue revealed a significantly increased expression of pro-inflammatory cytokines *Il1β* and *iNos* (Figure 5b).

Figure 5

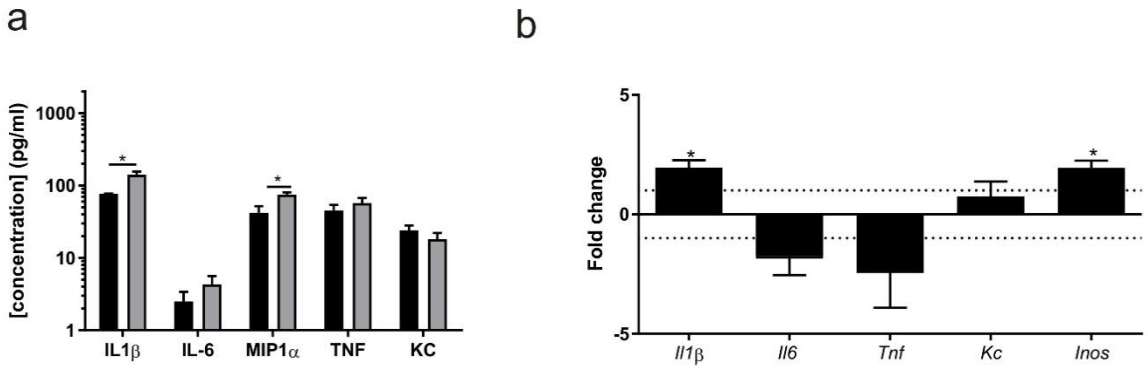


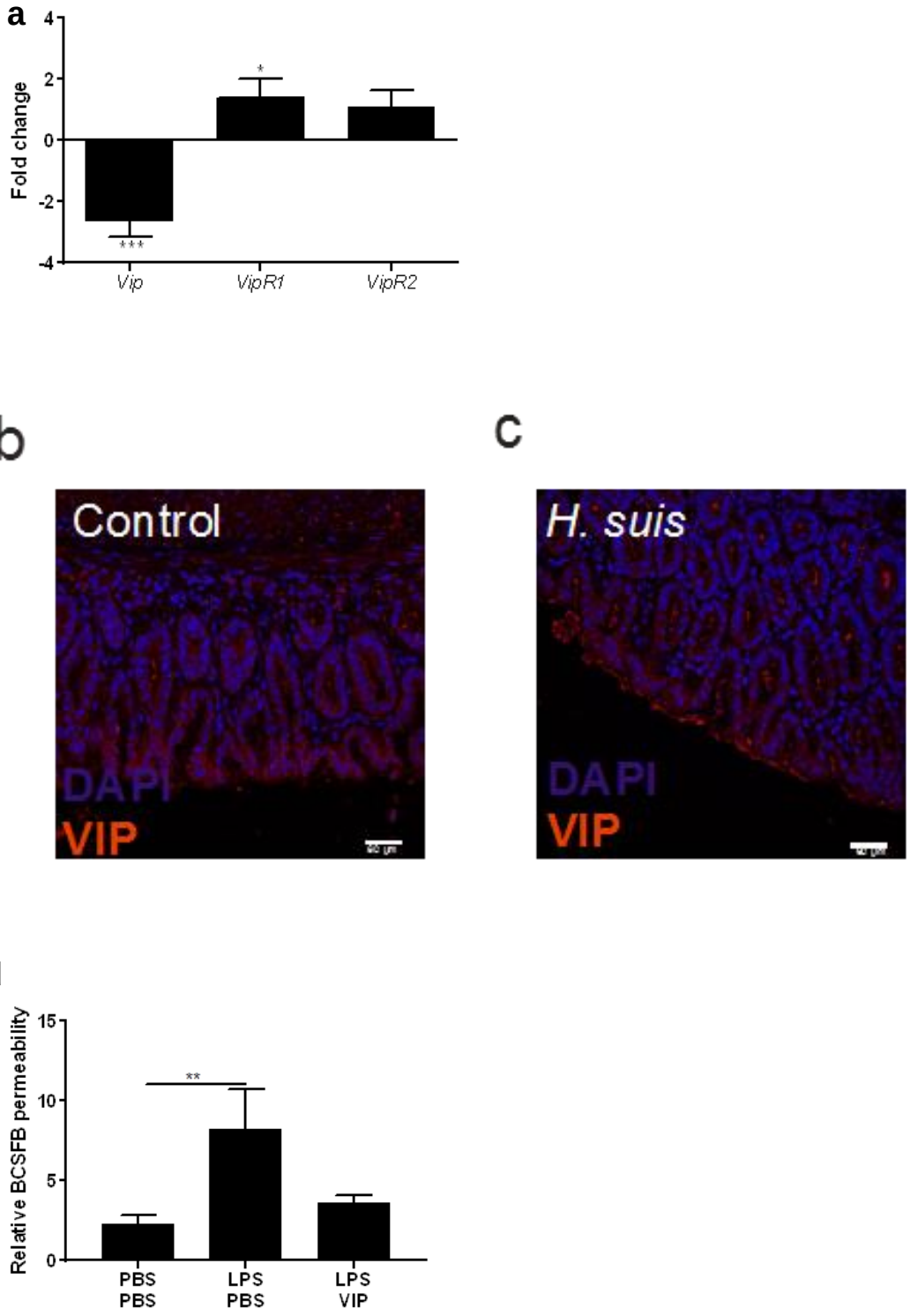
Figure 5. Analysis of systemic inflammation after one month of *Helicobacter suis* (*H. suis*) infection. (a) Levels of serum interleukin (IL) 1β, IL6, macrophage inflammatory protein (MIP1α), tumor necrosis factor (TNF) and keratinocyte chemoattractant (KC) determined using Bioplex after inoculation of mice with *H. suis* or growth medium,. Data were analyzed by Student's t-test (n = 3-4). **(b)** Expression of the inflammatory genes *Il1β*, *Il6*, *Tnf*, *Kc* and *Inos* in choroid plexus tissue of C57BL/6 mice inoculated with *H. suis* or growth medium, represented as fold change (n = 3-5).

H. suis infection disturbs VIP signaling

Another important link between the gastrointestinal system and the brain are neuropeptides, such as the vasoactive intestinal peptide (VIP) (De Vadder et al., 2015). Indeed, VIP seems not only to be involved in the maintenance of the gastrointestinal epithelial barrier integrity (Conlin et al., 2009) but might also play a role in brain homeostasis (Delgado et al., 2004). Additionally, VIP reactive neurons have been found in the enteric nervous system and in the choroid plexus (Lindvall et al., 1978; Delgado et al., 2004). One month after infection with *H. suis*, the expression of *Vip* in the corpus of the stomach was significantly decreased (Figure 6a), whereas expression of the receptor, *Vipr1*, was significantly up-regulated and *Vipr2* remained stable (Figure 6a). Additionally, immunofluorescent stainings revealed that VIP was retained in the lumen of the gastric glands of *H. suis*-infected animals (Figure 6b-c). To evaluate whether alterations in VIP levels could affect the blood-CSF barrier integrity and thereby brain homeostasis in a LPS-induced systemic inflammation model, mice were treated with VIP. As in the infection with *H. suis,* injection of LPS in mice resulted in increased leakage of the blood–CSF barrier as described before (Vandenbroucke et al., 2012) (Figure 6d); this was reversed by VIP treatment (Figure 6d). LPS-induced increased leakage of the blood–CSF barrier was associated with significant down-regulation of the tight junction proteins *Cldn5*, *Ocln* and *Zo3* in the choroid plexus (Figure 6e). Downregulation of *Ocln* was significantly less in LPS-injected mice treated with VIP (Figure 6e). Expression of *Cldn1* was increased significantly by LPS injection, whereas expression of *Zo1* decreased when LPS and VIP were combined (Figure 6e). LPS injection also increased inflammatory gene expression in the choroid plexus,

reflected by an increase of *Il1β, Il6*, *Tnf, Kc* and *iNos*; the increases in *Il6*, *Tnf, Kc* and *iNos* were significantly reduced by VIP treatment (Figure 6f). Together, these data indicate that VIP might play a protective role in the preservation of brain homeostasis in the presence of systemic inflammation, and it might be important in the *H. suis*-associated changes in the brain.

Figure 6



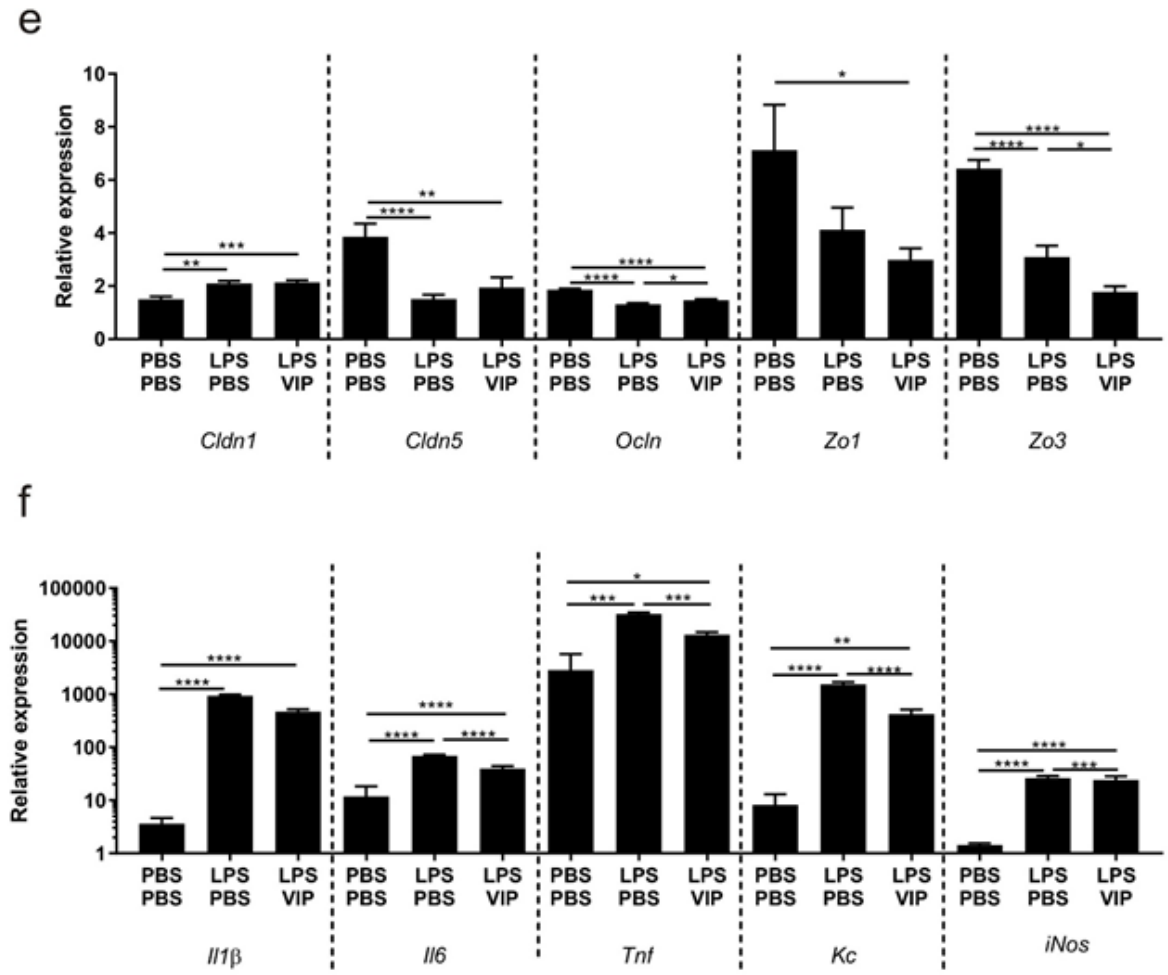


Figure 6. Analysis of vasoactive intestinal peptide (VIP) signaling after one month of *Helicobacter suis* (*H. suis*) infection. **(a)** Expression of vasoactive intestinal peptide (*Vip*), VIP receptor (*VipR1*) and *VipR2* in the corpus of the stomach of C57BL/6 mice inoculated with *H. suis* or growth medium, represented as fold change (n = 6-10). **(b-c)** Representative confocal images of VIP staining in the stomach of control (d) and *H. suis* infected (e) C57BL/6 mice. **(d)** Relative blood–cerebrospinal fluid (CSF) barrier permeability as a result of systemic inflammation induced by intraperitoneal (ip) injection of 120 µg/ 20 g body weight lipopolysaccharide (LPS), followed by intravenous (iv) injection of PBS or VIP, compared to controls (n = 5-8). **(e)** Relative expression of the tight junction proteins Claudin 1 (Cldn1), *Cldn5*, Occludin (*Ocln*), Zonula occludens 1 (*Zo1*) and *Zo3* in the choroid plexus as a result of systemic inflammation, followed by iv injection of PBS or VIP, compared to controls (n = 8-10). **(f)** Relative expression of the inflammatory cytokines interleukin 1β (*Il1β*), *Il6,* tumor necrosis factor (*Tnf*), keratinocyte chemoattractant (*Kc*) and inducible nitric oxide synthase (*iNos*) in the choroid plexus as a result of systemic inflammation, followed by iv injection of PBS or VIP, compared to controls (n = 8-10)

Discussion

Alterations in the gut microbiome are believed to contribute to many different human health problems (Parracho et al., 2005; Bercik et al., 2011; Bravo et al., 2011; Clemente et al., 2012; Zhang et al., 2015). Next to the direct effects *H. pylori* infection on the gastrointestinal system (Kusters et al., 2006; Mayer et al., 2014; Li et al., 2016), it has been associated with the occurrence of different neurodegenerative disorders, including Alzheimer's disease (Wirdefeldt et al., 2011; Alvarez-Arellano and Maldonado-Bernal, 2014; Wong et al., 2014) and Parkinson's disease (Wirdefeldt et al., 2011; Blaecher et al., 2013; Alvarez-Arellano and Maldonado-Bernal, 2014; Wong et al., 2014). We recently highlighted in a similar way the importance of gastric *H. suis* infections in Parkinson's disease (Blaecher et al., 2013). However, it remains unknown how gastric *Helicobacter* infection in general influences the brain.

The gastrointestinal system and the brain are connected through neural, endocrine and immune pathways. Here, we demonstrated that one month of infection with *H. suis* resulted in increased brain inflammation. In particular, we observed a moderate but significant up-regulation of *Il6* gene expression in the hippocampus, elevated levels of sTNFR1 in CSF (a biomarker for Alzheimer's disease) (Jiang et al., 2011), and an increased amount of activated microglia in the brain. Interestingly, elevated brain levels of IL6 have also been reported in traumatic brain injury (Goodman et al., 1990; Kossmann et al., 1995), depression (Brietzke et al., 2009), Parkinson's disease (Boka et al., 1994) and Alzheimer's disease (Angelopoulos et al., 2008), suggesting a role for IL6 in the above-mentioned neuropathologies. Furthermore, microglia activation has been observed in *H. pylori* infection (Lo et al., 2009), and priming of the microglia by prior pathology accelerates the pathology in the brain (Cunningham, 2013). To ensure homeostasis in the CNS, the brain is protected from fluctuations in the systemic blood composition by a series of brain barriers, including the BBB and the blood-CSF barrier (De Bock et al., 2014). Our results indicate that gastric *H. suis* infection can induce leakage of the blood–CSF barrier but not of the BBB. This disturbance was accompanied by loss of tight junctions, and specifically by a significant decrease in the expression of *Cldn1* and *Ocln* and disturbed localization of CLDN1 protein in the choroid plexus epithelium.

Although the observed *H. suis*-induced blood-CSF barrier leakage was rather limited, dysfunction of the choroid plexus epithelium is known to be destructive to the brain (Redzic et al., 2005; Marques et al., 2007; Johanson et al., 2011; Balusu et al., 2016a). Moreover, increased blood-CSF barrier permeability has been observed in several neuroinflammatory disorders, including Alzheimer's disease, aging and Parkinson's disease (Pisani et al., 2012; Marques et al., 2013; Brkic et al., 2015; Balusu et al., 2016a; Gorle et al., 2016). The choroid plexus epithelium is uniquely positioned between blood and brain, and we and others believe that this blood–CSF interface is an essential player in the communication between the periphery and the brain. Indeed, several studies reported that choroid plexus epithelial cells sense inflammatory changes in the periphery, which in turn induces changes in functionality of the choroid plexus epithelial cells (Thouvenot et al., 2006; Marques et al., 2007; Marques et al., 2009; Vandenbroucke et al., 2012; Balusu et al., 2016b). Also in our study, we observed *H. suis*-induced systemic inflammation characterized by increased levels of IL1β and MIP1α in the serum, which could have contributed to disruption of the blood-CSF barrier and subsequently to brain inflammation.

Disruption of the gastric mucosal barrier might lead to leakage of inflammatory cytokines, chemokines and bacterial components from the stomach into the systemic circulation (Cryan and Dinan, 2012; Mayer et al., 2014), thereby inducing systemic inflammation. In our study, the extent of colonization of the stomach by *H. suis* was similar to what has been reported (Flahou et al., 2010). DNA from *H. suis* was also found in the duodenum, but it is not clear whether this pathogen colonized the duodenum or, otherwise, the qRT-PCR picked up DNA from bacteria colonizing the stomach. Furthermore, *H. suis* infection evoked a pro-inflammatory immune response in the stomach characterized by infiltration of innate immune cells and up-regulation of IL1β and murine IL8 homologue genes *Lix* and *Kc*; these observations are in line with previous studies (Flahou et al., 2010; Flahou et al., 2012; Bosschem et al., 2016; Bosschem et al., In submission). Inflammation in the stomach was also accompanied by increased gastrointestinal barrier permeability and changes in the localization of the tight junction protein zonula occludens 1 (ZO1). Concordant with our study, co-culture of primary human gastric epithelial cells with *H. pylori* resulted in disruption of membrane ZO1 (Wrobleski, 2011).

Aberrant expression of transmembrane mucins has also been shown to play a role in disturbance of mucosal barrier integrity (Kufe, 2009). In our study, an inappropriate overexpression of the membrane-associated mucin MUC13 was seen. This mucin, which is predominantly expressed in the intestine and only at very low level in the stomach, seems to be induced by IL1β in the inflamed stomach during *Helicobacter* infection (Cheng et al., 2016). Inappropriate overexpression of transmembrane mucins can affect gastric mucosal barrier integrity by disrupting cell polarity and cell-cell interactions (Kufe, 2009), but further investigations are necessary to confirm the role of MUC13 in gastric mucosal barrier dysfunction. Taken together, gastric *H. suis* infection induced inflammation in the stomach, which was associated with gastric mucosal damage resulting in leakage of inflammatory mediators into the blood and leading to systemic inflammation.

The gastrointestinal tract and the brain communicate through the systemic pathway, but they might also interact via neural communication (Mayer et al., 2014). Vasoactive intestinal peptide (VIP) is a neuropeptide with diverse functions and was recently identified as a component of the gut-brain axis (De Vadder et al., 2015). VIP has been shown to promote homeostasis of the mucosal barrier of the gastrointestinal tract and has been implicated in tight junction regulation (Conlin et al., 2009; Chen et al., 2015). In our study, VIP expression was changed in the stomach of *H. suis* infected animals, which agrees with the previously described altered density of VIP immunoreactive nerves in mice in response to *H. pylori* infection (Bercik et al., 2002). Alterations in VIP expression might thus also contribute to dysfunction of the gastric mucosal barrier seen in our study.

In the brain, VIP can prevent overactivation of microglia in LPS-induced inflammation (Kim et al., 2000; Delgado and Ganea, 2003). VIP has been described as protective in Parkinson's disease (Delgado and Ganea, 2003) and Alzheimer's disease (Song et al., 2012). To determine whether alterations in the peripheral VIP levels affect the blood-CSF barrier during peripheral inflammation, mice were injected in the peritoneum with LPS and then treated with VIP. Our results indicate that administration of VIP in LPS-induced disruption of brain homeostasis could reverse inflammation in the choroid plexus. VIP reactive nerves had been demonstrated in the choroid plexus (Lindvall et al., 1978) and their contribution to the regulation of choroid plexus activity had been

described (Nilsson et al., 1991). These reports are in line with our finding that administration of VIP reduces LPS-induced leakage of the blood–CSF barrier.

In conclusion, we have identified the pivotal role of the choroid plexus in the communication between the gastrointestinal tract and the brain. Our results indicate that disruption of the blood-CSF barrier is induced by *H. suis*-associated systemic inflammation and/or dysregulated VIP signaling. Although further research is required to unravel the specific mechanisms of gastrointestinal–brain communication, VIP is a potential candidate for treatment of neurodegenerative diseases because it can restore the blood-CSF barrier and thereby reduce the neuroinflammation associated with gastrointestinal infections.

Acknowledgments

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Tables

Table 1. Overview of used qRT-PCR primer sequences.

	Forward primer sequence	**Reverse primer sequence**
H2afz	GGTATCACCCCTCGTCACTT	TCAGCGATTTGTGGATGTGT
Hprt **(stomach)**	CAGGCCAGACTTTGTTGGAT	TTGCGCTCATCTTAGGCTTT
Ppi	AGCATACAGGTCCTGGCATC	TTCACCTTCCCAAAGACCAC
Il1ß	CACCTCACAAGCAGAGCACAAG	GCATTAGAAACAGTCCAGCCCATAC
Kc	GCTGGGATTCACCTCAAGAA	TCTCCGTTACTTGGGGACAC
Mip2	TGCCTGAAGACCCTGCCAAGG	GTTAGCCTTGCCTTTGTTCAG
Lix	CTCAGTCATAGCCGCAACCGAGC	CCGTTCTTTCCACTGCGAGTGC
Ocln	CCAGGCAGCGTGTTCCT	TTCTAAATAACAGTCACCTGAGGGC
Cldn1	TCTACGAGGGACTGTGGATG	TCAGATTCAGCAAGGAGTCG
Cldn5	GCAAGGTGTATGAATCTGTGCT	GTCAAGGTAACAAAGAGTGCCA
Muc5ac	Purchased from Qiagen (Qt01196006)	
Muc5b	CAGATCCATCCATCCCATTTCT	TATCTGACTACCACTTGTTGATGTGACT
Muc6	TGCTCCCAGAATGAGTACTTCGA	CAGAGGTGGAACTGTGAAACTCAGT
Muc13	GCCAGTCCTCCCACCACGGTA	CTGGGACCTGTGCTTCCACCG
Vip	AGTGTGCTGTTCTCTCAGTCG	GCCATTTTCTGCTAAGGGATTCT
VipR1	GATGTGGGACAACCTCACCTG	TAGCCGTGAATGGGGGAAAAC
VipR2	GACCTGCTACTGCTGGTTG	CAGCTCTGCACATTTTGTCTCT
Tnf	ACCCTGGTATGAGCCCATATAC	ACACCCATTCCCTTCACAGAG
iNos	CAGCTGGGCTGTACAAACCTT	CATTGGAAGTGAAGCGTTTCG
Zo1	AGGACACCAAAGCATGTGAG	GGCATTCCTGCTGGTTACA
Zo3	ACCCTATGGCCTGGGCTTC	CCCGGGTACAACGTGTCC
Hprt choroid plexus	AGTGTTGGATACAGGCCAGAC	CGTGATTCAAATCCCTGAAGT
Rpl	CCTGCTGCTCTCAAGGTT	TGGTTGTCACTGCCTGGTACTT
Ubc	AGGTCAAACAGGAAGACAGACGTA	TCACACCCAAGAACAAGCACA

Gapdh	TGAAGCAGGCATCTGAGGG	CGAAGGTGGAAGAGTGGGAG
H. suis	AAAACAMAGGCGATCGCCCTGTA	TTTCTTCGCCAGGTTCAAAGCG

Chapter 3

A novel isolation protocol and probe-based RT-PCR for diagnosis of gastric infections with the zoonotic pathogen *Helicobacter suis*

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Abstract

Background: *Helicobacter suis* is a very fastidious micro-organism associated with gastritis, gastric ulcers and mucosa-associated lymphoid tissue (MALT) lymphoma in humans. *In vitro* isolation of this agent from human patients has so far been unsuccessful.

Materials and methods: A probe-based real-time (RT-) PCR for the rapid detection of *H. suis* in gastric biopsies was developed. Secondly, a mouse-passage-based protocol was optimized for isolation of low numbers of viable *H. suis* bacteria. Mice were inoculated with different numbers of viable *H. suis* (10^2-10^8) and kept for 4 weeks to allow multiplication of this pathogen.

Results: The probe-based real-time (RT)-PCR exhibited a high degree of diagnostic specificity and analytical sensitivity, high linear correlations (r^2 between 0.995 and 0.999) and high amplification efficiencies (> 90%) for *H. suis.* No cross-reactivity was detected with human, porcine, non-human primate and murine DNA nor with DNA from other bacteria including *Helicobacter* spp. and *Campylobacter* spp.

H. suis was successfully re-isolated from the stomach of mice inoculated with at least 10^4 viable *H. suis*, using a biphasic medium (pH 5), consisting of *Brucella* agar with *Brucella* broth on top, both supplemented with vitox supplement, Campylobacter-selective supplement, amphotericin (5 µg/ml), HCl (0.05 %), fetal bovine serum (20 %) and linezolid (5 µg/ml). Linezolid was necessary to inhibit proliferation of contaminants, including lactobacilli.

Conclusion: The methods described in this paper can be implemented for detection or isolation of *H. suis* from human gastric biopsies.

Introduction

Helicobacter pylori (*H. pylori*) is associated with a wide range of gastric disorders, including gastritis, ulcers and cancer (Montecucco and Rappuoli, 2001). However, gastric disease in humans has also been associated with other *Helicobacter* species. These are collectively called non-*H. pylori Helicobacter* spp (NHPH). They are long, spiral-shaped organisms most commonly colonizing the stomachs of domestic animals (Haesebrouck et al., 2009). Reported estimates of prevalence of NHPH in human populations range from 0.2 to 6 % (Haesebrouck et al., 2009). However, due to difficulties in the diagnosis of NHPH infections these estimates are certainly biased and most probably underestimate the true prevalence. Moreover, it cannot be excluded that infections with these agents sometimes remain asymptomatic or cause only mild symptoms which are not rigorously examined. The prevalence of *H. pylori* in the Western world is decreasing, leaving a niche for infection with NHPH (Haesebrouck et al., 2009; Overby et al., 2016).

H. suis appears to be the most important amongst the NHPH. Not only does it represent the most common of the NHPH detected in the stomach of people suffering from gastric disorders, but it has also been detected in 27 % of Parkinson's disease (PD) patients and only in 2 % of controls from gastroenterology-services using an *ureA*-based SYBR green RT-PCR assay on gastric biopsies with positivity on amplicon sequencing (Blaecher et al., 2013).

Pigs are the main reservoir of *H. suis* (Haesebrouck et al., 2009; De Bruyne et al., 2012), but *H. suis* has also been found in the stomach of rhesus and crab-eating macaques (O'Rourke et al., 2004; Martin et al., 2013; Bosschem et al., 2016). *H. suis* is transmitted to humans most likely through direct or indirect contact with pigs (Joosten et al., 2013). Additionally, this bacterium can be present and persist in minced pork, suggesting that raw or undercooked pork may also constitute a source of *H. suis* infections in humans (De Cooman et al., 2013).

H. suis has been associated with gastritis, peptic and duodenal ulcers, and low-grade mucosa-associated lymphoid tissue (MALT) lymphoma in humans. Compared to *H. pylori*, gastritis caused by *H. suis* appears less active and less severe, but the risk of developing MALT lymphoma is higher (Nakamura et al., 2007; Nakamura et al., 2008; Matsui et al., 2014).

The diagnosis of gastric *Helicobacter* infections is extremely difficult and usually involves endoscopic sampling of gastric biopsies. Commercial non-invasive tests to specifically diagnose infections with *H. suis* are currently unavailable. Attempts to isolate *H. suis* from human gastric biopsies, either directly, or after mouse-passage (Matsui et al., 2014) have been unsuccessful so far. Direct isolation from biopsies may be hampered by the low colonization density of this agent (Blaecher et al., 2013), whilst isolation after mouse-passage has been hampered by overgrowth of *Lactobacillus* spp. (Matsui et al., 2014; Aiba et al., 2015). Analysis of the gastric biopsies by molecular methods currently appears to be the only feasible way to identify infections with *H. suis*. One available molecular method makes use of RT-PCR based on the *ureA* gene for identification and quantification of *H. suis* bacteria (Blaecher et al., 2013; Flahou et al., 2010). In this assay, additional sequencing of the positive PCR amplicons is necessary, since cross-reactivity with host DNA and DNA from other gastric NHPH may occur (unpublished results). Recently, a new *H. suis*–specific PCR has been developed, using primer pairs targeting the *carR* gene. Compared to *ureA*, this *carR* gene is a good candidate for the identification of *H. suis*, since it is not present in other gastric *Helicobacter* genomes (Matsui et al., 2013). However, this novel

assay required further investigation, as it is not known whether the *carR*-specific primers react with other bacterial or host DNA.

The purpose of the present study is to further improve diagnostic tests for the detection of *H. suis*. We first developed and validated a *carR* probe-based RT-PCR that allows detection and quantification of *H. suis* in gastric tissue samples. Secondly, a mouse-passage-based protocol was further optimized for the isolation of low numbers of *H. suis* bacteria. Since mouse-passage of *H. suis* might influence strain characteristics, we also sequenced the genome of a porcine *H. suis* strain before and after passage in mice.

Materials and methods

1) *CarR* probe-based RT-PCR

Bacterial strains and *in vitro* culture conditions

H. suis , *H. heilmannii*, *H. ailurogastricus* and *Lactobacillus* spp. (*L. reuteri* and *L. gasseri*) (Table 1) were cultivated biphasically on *Brucella* agar (Immunosource, Halle-Zoersel, Belgium) plates, supplemented with 1 ml Skirrow (Oxoid, Hampshire, United Kingdom), 3 ml Vitox (Oxoid), 5 µg/ml amphotericin B (Sigma-Aldrich, Steinheim, Germany), 0.05 % HCL (Sigma-Aldrich) and 20 % inactivated fetal bovine serum (Perbio, Thermo Scientific Hyclone, Erembodegem; Belgium) (*H. suis* growth medium) (Baele et al., 2008) *H. bizzozeronii, H. felis, H. salomonis, H. pylori, H. cynogastricus, H. baculiformis, H. cetorum, H. cholecystus, H. trogontum, H. acinonychis, H. mustellae, H. pullorum, H. marmotae, H. mesocricetorum, H. bilis, H. equorum, H. canadensis* and *Campylobacter* spp. (*C. jejuni, C. coli, C. fetus* and *C. upsaliensis*) (Table 1) were grown on brain heart infusion (BHI) agar (Bio-rad, Nazareth, Belgium) plates supplemented with 10 % horse blood (E&O Laboratories, Bonnybridge, UK), Skirrow, Vitox, and 5 µg/ml amphotericin B. All *Helicobacter* spp. and *Campylobacter* spp. were cultivated under micro-aerobic conditions (10 % CO_2, 5 % O_2 and 85 % N_2; 37°C). *Staphylococcus aureus, Staphylococcus pseudintermedius, Streptococcus suis, Streptococcus equi* subsp. *equi*, *Streptococcus equi* subsp. *zooepidemicus, Pasteurella multocida, Mannheimia haemolytica, Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus vulgaris, Bacillus licheniformis, Salmonella* Typhimurium*, Trueperella pyogenes, Escherichia coli, Aeromonas hydrophila, Enterococcus faecalis* and *Actinobacillus equuli* were grown aerobically on commercially available Columbia agar plates supplemented with 5 % sheep blood (Oxoid) at 37°C. *Clostridium perfringens* and *Fusobacterium gastrosuis* (Table 1), grown on Columbia agar plates (with 5% sheep blood), were incubated anaerobically at 37°C, whereas *Yersinia pseudotuberculosis* was grown on Columbia agar plates (with 5 % sheep blood) and incubated aerobically at 30°C (Table 1).

Primer and probe design

Analysis and comparison of the *carR*-gene sequence from *H. suis* strains HS1 (Genbank accession no. ADGY00000000), HS5 (Genbank accession no. HDAO00000000), TKY (EMBL/Genbank/DDBJ database accession no. AB849014), SNTW101 (EMBL/Genbank/DDBJ database accession no. AB849015), HS8 (EMBL/Genbank/DDBJ database accession no. AB849016) and HS10 (EMBL/Genbank/DDBJ database accession no. AB849017) resulted in the selection of a specific 149 bp region. Based on this region (149 bp), primers and a probe (20 bp) were developed: sense primer *carR*-FQ1 (5'-TGT GGC TAG RTG TTT GCC -3'), antisense primer

carR-RQ1 (5'-CT GCA GGC AAY GCT GTT GTT -3') and *carR* probe (5'- ACC ACG CCT GCA GGC AAC AA-3'). The melting temperature and GC% of the primers and probe were calculated and optimized using the OligoAnalyser3.1 (Integrated DNA technologies, Leuven, Belgium). Subsequently, the primers and probe were verified using BLASTN to ensure that amplification of genes from other organisms or species was unlikely. Finally, the probe was combined with the dye 6-carboxyfluorescein (FAM) and the quencher ZEN/3IABkFQ.

Bacterial DNA extraction and quantitation of standards

DNA was extracted from the different bacterial species using the Prepman™ reagent (Life Technologies, Ledeberg, Belgium) according to the manufacturer's instructions. For generation of the standard for the RT-PCR assay, part of the *carR* gene (423bp) from *H. suis* HS5 was amplified using primers cbcarR-fq (5'- ATG TAC AAG SGC TCT TGA GGG -3') and cbcarR-rq (5'- GAT GGC TGC TTG GAA ACT AGC -3') with an annealing temperature of 57°C. Subsequently, the DNA concentration of the PCR products was measured and diluted to a concentration of 10^{10} Genomic equivalents (GE). The standard consisted of 10-fold-dilution series starting at 10^{9} GE for each 10 µl of reaction mixture.

***H. suis ureA*-based SYBR green RT-PCR**

The *H. suis* SYBR green assay based on the amplification of part of the gene encoding the urease A subunit was carried out as described before (Blaecher et al., 2013).

***H. suis carR* SYBR green RT-PCR**

The *H. suis carR* RT-PCR was first evaluated using SYBR green. Two µl of DNA template (samples and standard) was suspended in a 10 µl reaction mixture consisting of 3.5 µl HPLC water, 6 µl SensiMix™ SYBR No-ROX (Bioline Reagents Ltd, London, UK) and 0.25 µl of primers *carR*-FQ1 and *carR*-RQ1 located within the 423 bp fragment to yield a 149 bp PCR product. The amplification conditions consisted of 95°C for 15 min followed by 47 cycles of denaturation (30 seconds at 95°C), annealing (30 sec at 56°C) and elongation (30 sec at 72°C). The final elongation step took place at a temperature of 95°C for 15 min. Melting curve data were obtained starting at 65°C to 95°C with a velocity of 0.5°C per 5 sec. The standard and bacterial DNA samples were run in duplicate on a CFX384™ RT-PCR System or a CFX96™ RT-PCR System with a C1000 Thermal Cycler (Bio-Rad).

***H. suis carR* probe-based RT-PCR and optimization**

Assays were performed on a CFX96 real-time system (Bio-rad). Primer concentrations were determined in a checkerboard system with a standard probe concentration of 250 nM. Subsequently, the probe concentration was optimized with the previously determined optimal primer concentrations (Blooi et al., 2013). The reaction mixture of 12 µl consisted of 6 µl of IQ™ Supermix (Bio-rad), 3.25 µl of HPLC water (Merck Millipore, Overijse, Belgium), 0.3 µl of both primers (*carR*-FQ and *carR*-RQ), 0.15 µl of the probe and 2 µl DNA template (standards and samples). The amplification conditions consisted of 95°C for 10 min followed by 47 cycles of denaturation (20 sec at 95°C), annealing (30 sec at 62.5°C) and elongation (30 sec at 73°C). The final elongation step took place at a temperature of 95°C for 15 min. Both standards and samples were run in duplicate. The precision, i.e.

the reproducibility and repeatability of the developed *H. suis carR* probe-based RT-PCR was evaluated by determining inter- and intra-assay variability, both expressed by the standard deviation (SD) (Bustin et al., 2009). Intrarun variability was ascertained by comparing the obtained cq-values for the standard ten-fold dilution series (starting from 10^9GE) within one RT-PCR run, whereas interrun variability was evaluated by comparing those values between 3 RT-PCR runs. The diagnostic specificity of the *carR*-based SYBR green RT-PCR, the *carR* probe-based RT-PCR and the *ureA*-based SYBR green RT-PCR was evaluated by assaying DNA extracts of different *Helicobacter* spp*., Campylobacter* spp*.* and other bacteria (Table 1). The amplification efficiency, slope and r^2 were calculated by linear regression analysis using the Bio-Rad CFX Manager v1.6 software (Bio-Rad, Hercules, CA,USA).

Gastric tissue samples for validation of the *carR* probe-based RT-PCR

To validate the use of the *carR* probe-based RT-PCR to detect *H. suis* in gastric tissue samples and exclude reactivity with host DNA, we applied the optimized protocol to analyze 80 human biopsies in which *H. suis* infection was or was not detected by *ureA*-based SYBR green RT-PCR with positivity on amplicon sequencing (Blaecher et al., 2013; Table 2), 9 gastric samples from pigs with or without known natural *H. suis* infection (Table 2), 9 gastric samples from non-human primates with or without known natural *H. suis* infection (Bosschem et al., 2016; Table 2) and 8 gastric samples from mice experimentally inoculated with *H. suis* or with the growth medium of the bacterium (Table 2). The human gastric biopsies originated from a previous experiment with approval from the ethics committee of Ghent University (EC UZG 2011/393) and from the diagnostic service of the Gastrointestinal Reference Unit, Public Health England. The non-human primate gastric samples originated from rhesus macaques and crab-eating macaques, residing at the Biomedical Primate Research Centre (Rijswijk, The Netherlands), accredited by the association for Assessment and Accreditation of Laboratory Animal Care (EU Directive 63/2010), and which were euthanized for reasons unrelated to the study. The stomach samples from control mice and mice experimentally infected with *H. suis* originated from an experiment with approval from the ethics committee of the faculty of Veterinary Medicine, Ghent University (approval no. EC 2014/73). The gastric tissue samples from pigs, used in the study by De Witte and colleagues (De Witte et al., 2016), were included in the analyses as well.

DNA from all gastric tissue samples was extracted using the Qiagen Blood and Tissue kit (Qiagen, Venlo, The Netherlands), according to the manufacturer's instructions.

2) <u>Optimization of a mouse-passage-based isolation protocol for *Helicobacter suis*</u>

Mice and experimental procedure

Six-week-old, female specific pathogen free (SPF) C57BL/6JolaHsd mice were purchased from Harlan NL (Horst, The Netherlands). The animals were housed in individual filter top cages, had free access to water and food (an autoclaved commercial diet: TEKLAD 2018S, containing 18 % protein; Harlan) throughout the experiment and were monitored daily. The experimental protocol was approved by the ethics committee of the faculty of Veterinary Medicine, Ghent university, Belgium (approval no. EC 2012/58).

H. suis HS1, grown biphasically as described above, was used as inoculum. After incubation under micro-aerobic conditions, the bacteria were harvested. Ten-fold dilution series were prepared in *Brucella* broth. The final concentrations ranged from 10^8 to 10^2 viable *H. suis* bacteria/ml. For each bacterial concentration, 2 mice were anaesthetized on two successive days with 2.5% isoflurane and then intragastrically inoculated with 300 µl using a feeding canula. At 4 weeks post-inoculation, animals were euthanized by cervical dislocation under deep isoflurane anaesthesia (5%). The stomach of each mouse was resected for isolation purposes.

Isolation of porcine *H. suis* strain from the murine stomach after mouse-passage

The *H. suis* isolation protocol, which has successfully been applied on porcine and non-human primate stomachs highly colonized with *H. suis* (Baele et al., 2008; Bosschem et al., 2016), was further optimized for use on murine gastric tissue with low numbers of *H. suis* bacteria. We found that supplementation with linezolid at 5 µg/ml to both phases of the *H. suis* growth medium did not inhibit *H. suis* cultivation, whereas the growth of contaminants present in the murine stomach was prevented (unpublished results).

This 5 µg/ml linezolid containing selective medium was evaluated in the experimentally infected mice. The murine stomachs were opened via the greater curvature. The gastric content was removed and the stomachs were washed and submerged in a 1 % HCl solution for 45 min. Each stomach was homogenized and half of it was inoculated onto the linezolid-supplemented *H. suis* growth medium, whereas the other half was inoculated onto the *H. suis* growth medium without linezolid supplementation (control sample). Subsequently, approximately 150 µl *Brucella* broth with or without (control sample) supplementation of 5µg/ml linezolid was added on top of the inoculum. The plates were incubated at 37°C under micro-aerobic conditions, daily checked for growth and humidified with the linezolid-supplemented *Brucella* broth or the *Brucella* broth without linezolid (control samples). Finally, pure isolates were transferred to *H. suis* growth medium without linezolid and stored at -70°C until further use.

Whole genome sequencing of *H. suis* isolates obtained after mouse-passage

The genomic DNA of 4 isolates obtained after mouse-passage of the porcine *H. suis* HS1 strain (isolate HS1_output (O)1, isolate HS1_O2, isolate HS1_O3 and isolate HS1_O4, originating from 4 different mice) and the original porcine HS1_input (I)1 isolate, retrieved from the pig's stomach and used for mouse-passage, was extracted using the Qiagen Blood and Tissue kit (Qiagen) according to the manufacturer's guidelines. Whole genome sequencing was performed as described before (Joosten et al., 2015). Briefly, 1 ng genomic DNA was used for library generation. Sequencing libraries were then prepared using Nextera XT chemistry (Illumina Inc., San Diego, CA, USA) in accordance with the manufacturer's recommendations. Subsequently, libraries were sequenced for a 250-300 bp paired-end sequencing run using the Miseq personal sequencer (Illumina). All genomes were assembled with the CLC Genomics Workbench, version 7 and annotated using the RAST server (Aziz et al., 2008).

Identification of recombination events in the genomes of *H. suis* isolates obtained after mouse-passage

Homologous recombination in 4 *H. suis* isolates obtained after mouse-passage of the porcine *H. suis* strain HS1 was assessed using an iterative application of ClonalFrameML as follows (Didelot & Wilson, 2015). From the obtained genome assemblies, a core genome alignment was first created using the genome profiler (GeP) software tool (Zhang et al., 2015). Subsequently, a maximum-likelihood input phylogeny was inferred using RaxML v7.0.43 (Stamakis, 2006) under the General Time-Reversible model. Both the RaxML tree and the core genome alignment were then analyzed by ClonalFrameML. This assay was rerun several times until the number of recombination events and phylogeny remained stable. Estimates of recombination parameters and the set of recombination events were recorded from the final run.

Nucleotide sequence accession numbers

The genome sequences determined in this study have been deposited in the EMBL database under BioProject record numbers: PRJEB15615, PRJEB15616, PRJEB15617, PRJEB15618, PRJEB15619 and received the following accession numbers: FMSU01000000, FMSQ01000000, FMSS01000000, FMST01000000 and FMSR01000000.

RESULTS AND DISCUSSION

1) *CarR* probe-based RT-PCR

***CarR* probe-based RT-PCR assay optimization**

The primer and probe concentrations used for this assay were the lowest concentrations that yielded the lowest quantitation cycle (cq) value and the highest ΔR_n. This latter is the R_n value (the fluorescent emission of the reporter dye, normalized to the background fluorescence) of the complete reaction mixture (including template) minus the R_n value of a negative control (Blooi et al., 2013). This resulted in a final reaction mixture of 12µl composed of 6µl of IQ™ Supermix (Bio-rad, Belgium), 3.25 µl of HPLC water (Merck Millipore, Belgium), *H. suis*-specific *carR* forward primer *carR*-FQ1 at a concentration of 0.5 µM, *H. suis*-specific *carR* reverse primer *carR*-RQ1 at a concentration of 0.5 µM, *H. suis*-specific *carR* FAM-labeled probe at a concentration of 0.25 µM and 2 µl template. By application of a temperature gradient for analysis of the standard curve, the optimal annealing temperature for the combination of *carR*-FQ1, *carR*-RQ1 and the *carR* probe was set at 62.5°C, leading to a run amplification efficiency between 90-100%, the square of the linear correlation (r^2) between 0.995 and 0.999 and a slope of -3.53 (Figure 1). With the exception of a change in the annealing temperature, the amplification conditions of the *carR* probe-based RT-PCR were identical to the conditions used in the *H. suis ureA*-based SYBR green RT-PCR.

In 2004, Schabereiter-Gurtner and co-workers (Schabereiter-Gurtner et al., 2004) developed an *ureA* probe-based RT-PCR and a 23S rRNA probe-based RT-PCR for the detection of *H. pylori* in stool samples. In contrast to our developed *carR* probe-based RT-PCR assay, no information on the linear correlation of the *ureA/*23S rRNA probe-based RT-PCR assays for the detection of *H. pylori* was provided in this study. However for these latter assays, a slope of -3.7 was reported for both standard curves, consisting of tenfold dilution series. Using the formula, E=10^-(1/slope)-1, an efficiency of 86 % could be calculated for both the *ureA* and 23S rRNA probe-

based RT-PCR assays. Since the *carR* probe-based RT-PCR in our study resulted in a slope of -3.53 and the optimal slope for a tenfold dilution series is considered -3.32 (Real-time PCR application guide, Bio-Rad Laboratories, 2006), the *carR* probe-based RT-PCR is characterized by a higher amplification efficiency compared to the *ureA/*23S rRNA probe-based RT-PCRs. As amplification efficiency is a measure for the percentage of DNA template amplified in each cycle and a slope of -3.32 for a tenfold dilution series equals an amplification efficiency of 100% (Real-time PCR application guide, Bio-Rad Laboratories, 2006), the amplification of DNA template is more efficient in the *carR* probe-based RT-PCR assay for the detection of *H. suis* compared to the *H. pylori*-specific *ureA/*23S rRNA probe-based RT-PCR assays.

Figure 1

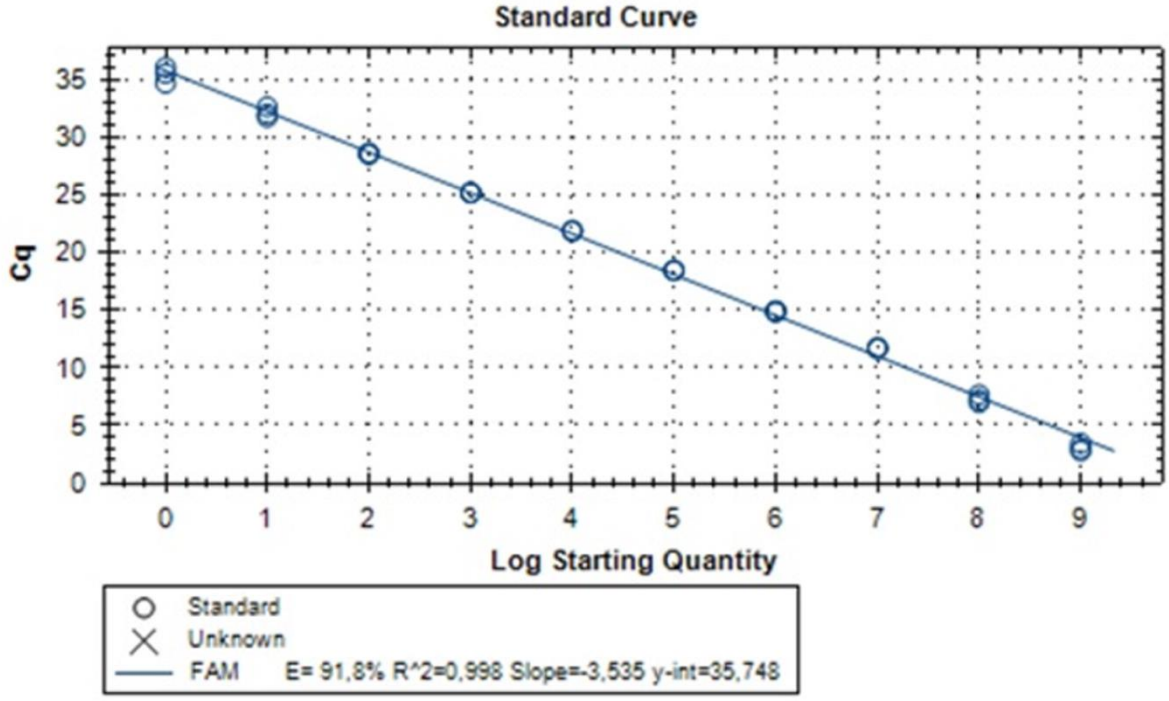

Figure 1: Standard curve along with efficiency and linear correlation of *carR* probe-based RT-PCR. Standards were run in duplicate. The *carR* probe-based RT-PCR is characterized by a high efficiency (>90 %) and a linear correlation (r^2) between 0.995 and 0.999.

Analytical sensitivity and diagnostic specificity

The analytical sensitivity of the *carR* probe-based RT-PCR was tested with the described quantitation standard of *H. suis*. Triplicates of serial dilutions ranging from 10^0 to 10^9 GE were assayed. In all replicates, cq-values were generated, suggesting that the limit of detection (LOD) of *H. suis* DNA using the *carR* probe-based RT-PCR assay is 1 GE per PCR. In theory, a DNA dilution of 10^0 will contain 1 GE per PCR. However, Bustin (Bustin et al., 2009) highlighted that the most sensitive LOD is 3 GE per PCR assuming a Poisson distribution, a 95% chance of including at least 1 GE per PCR and 1GE detection (Bustin et al., 2009). This means that in case of a dilution of 10^0 GE, 37 % of the aliquots will contain no GE, another 37% will contain 1 GE and the final 26 % will contain 2 or more GE (Bustin et al., 2009). A similar detection limit has been reported for the *H. suis*-specific *ureA*-based SYBR green RT-PCR (Blaecher et al., 2013; Bosschem et al., 2015).

To verify the diagnostic specificity of the *carR* probe-based RT-PCR, DNA samples from several bacteria (Table 1), including different *Helicobacter* spp., *Campylobacter* spp. and members of the *Enterobacteriaceae* family (Schabereiter-Gurtner et al., 2004; Moyaert et al., 2008; Matsui et al., 2014), were analyzed. Of all 77 different bacterial non-*H. suis* samples and in contrast to the *ureA*-based SYBR green RT-PCR (Table 1), the *carR* probe-based RT-PCR amplified only *H. suis* DNA and not the DNA from the other 77 bacterial non-*H. suis* samples (95 % confidence interval: 95%-100%), indicating a high degree of diagnostic specificity. No aspecific reactions with DNA from gastric and enterohepatic *Helicobacter* spp., *Campylobacter* spp. and other bacteria were observed (Table 1). Identical results were obtained using the *carR*-based SYBR green RT-PCR, highlighting the diagnostic specificity of the *carR* primers for *H. suis*.

Assay performance and precision

To determine the assay performance and precision of the *carR* probe-based RT-PCR with the described quantitation standards of *H. suis*, intrarun and interrun variability was defined as described before (Blooi et al., 2017). Both inter- and intrarun assays of the *carR* probe-based RT-PCR were characterized by high squared linear correlation (r^2> 0.995) and amplification efficiency (≥90 %) values, together with low intra- and inter-assay variabilities (SD < 1; Figures 2 and 3). The cq-values for the dilution series of *H. suis* bacteria in a run and between 3 different runs were similar (Figure 2 and 3). Within a run the highest variation was observed at 10^9 GE, resulting in a standard deviation of 0.97 (Supplementary Table 1), whereas between different runs the highest variation was observed at 10^0 and 10^9 GE, resulting in a standard deviation of 0.76 and 0.62, respectively (Supplementary Table 2). The separate cq-values of each inter- and intrarun assay are presented in Supplementary Table 1 and 2. The above results demonstrate that the probe-based RT-PCR has a high reproducibility as well as a good performance over the tested quantitation range.

Figure 2

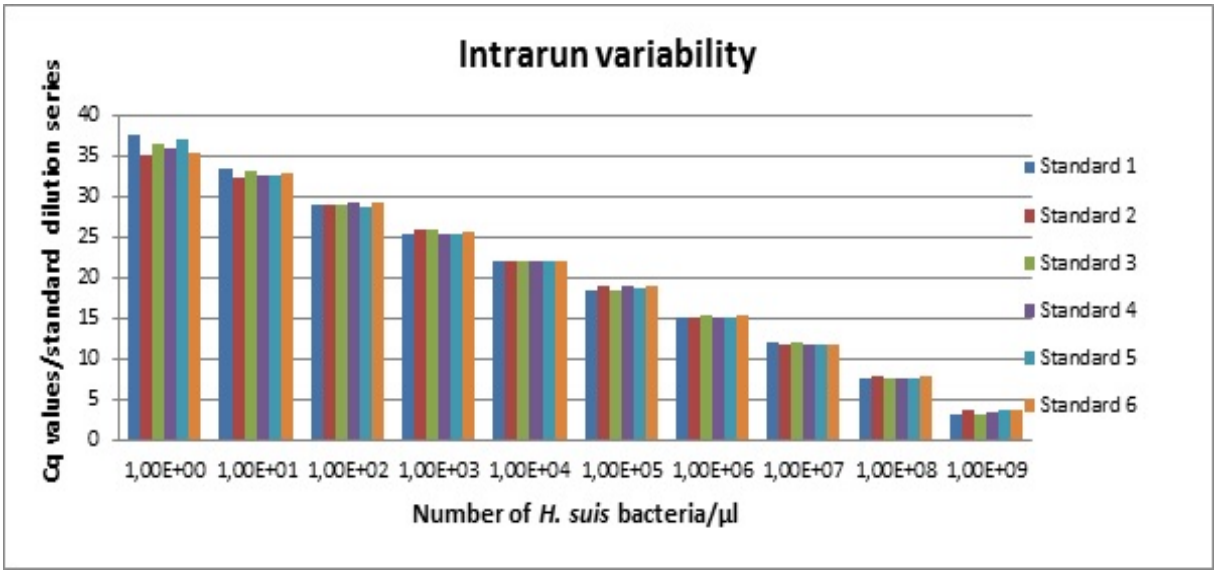

Figure 2: Overview of the cq-values of the standard dilution series in 1 run. The cq-values for the dilution series of *H. suis* bacteria within 1 run were similar. Inside 1 run the highest variation was observed at 10^9 GE, resulting in a standard deviation of 0.97. The separate cq-values of one run are demonstrated in Supplementary Table 2.

Figure 3

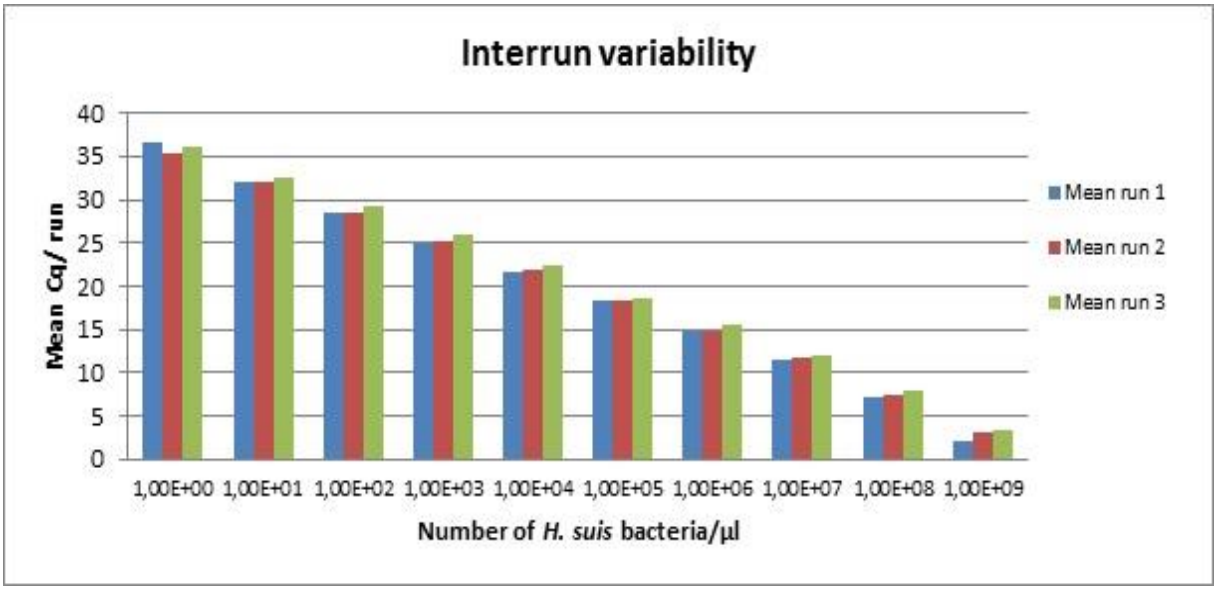

Figure 3: Overview of the mean cq-values of the standard dilution series between 3 different runs. Between 3 different runs, the cq-values were likewise the same as in 1 run. At the dilution of 10^9 and 10^0 GE, the highest standard deviation of the cq-values between the 3 runs amounted 0.76 and 0.62, respectively. The separate cq-values of the 3 different runs are demonstrated in Supplementary Table 3.

Validation on gastric tissue samples

To validate the developed *H. suis*-specific *carR* probe-based RT-PCR, DNA from human, non-human primate, murine and porcine gastric tissue samples was assayed (Table 2 and Supplementary Table 3). All tested samples were run in the *H. suis*-specific *ureA*-based SYBR green, the *carR*-based SYBR green and the *carR* probe-based RT-PCR assay to compare variability in cq-values between the assays. Out of the 80 human gastric biopsy samples, the *ureA*-based SYBR green RT-PCR identified 7 positive human gastric samples. Six of these also reacted positive using the *carR* probe-based RT-PCR. None of the human gastric samples tested positive for *H. suis* DNA using the *carR*-based SYBR green RT-PCR (Table 2 and Supplementary Table 3). The number of *H. suis* positive non-human primate samples identified by the *ureA*-based SYBR green RT-PCR and the *carR* probe-based RT-PCR were 9 and 8, respectively, whereas only 6 of the 9 samples tested positive for *H. suis* DNA using the *carR*-based SYBR green RT-PCR (Table 2 and Supplementary Table 3). Finally, for both murine and porcine gastric tissue samples, all known negative and positive *H. suis* samples were properly identified using both *carR* probe-based RT-PCR and *ureA*-based SYBR green RT-PCR. However, none of the known positive murine and porcine gastric samples were identified using the *carR*-based SYBR green RT-PCR (Table 2 and Supplementary Table 3).

Additional sequencing was performed for the samples reacting positive in *ureA*-based SYBR green RT-PCR. One human and one non-human primate sample, which tested negative for *H. suis* using the *carR* probe-based RT-PCR, were false positive in the *ureA*-based SYBR green RT-PCR (Table 2 and Supplementary Table 3). The sequence of the amplicon obtained within the human gastric sample corresponded to that of human DNA. That

of the non-human primate gastric sample corresponded to the *ureA/B* gene sequence of an unknown *Helicobacter* sp.

In contrast, none of the human, porcine and murine samples tested positive for the presence of *H. suis* DNA using the *carR*-based SYBR green RT-PCR. Although the melting curve obtained for these human, porcine and murine samples did not correspond with that of *H. suis*, high cq-values were detected for these samples using the *carR*-based SYBR green RT-PCR. Sequencing of the amplicons obtained in the SYBR green RT-PCR with *carR*-based primers revealed that human, porcine, murine and primate DNA was amplified, indicating that these primers do not only bind to the *H. suis carR* gene, but also to host DNA which is present in large quantities in gastric samples. This renders the *carR*-based SYBR green RT-PCR detection of amplicons inefficient and a method that specifically detects DNA sequences amplified from the *H. suis carR* gene is required. In the present study, this was realized using a probe that detects a specific part of the *carR* gene. Indeed, the SYBR green RT-PCR assay emits a fluorescent signal when the fluorophore binds to any amplified double stranded DNA. In contrast, the probe binds on a specific region within the amplicon of single stranded DNA. The emission of fluorescence only takes place when the reporter dye is separated from the quencher by the DNA polymerase.

Since the true positive samples, derived from the human, non-human primate, murine and porcine stomachs, tested false negative using the *carR*-based SYBR green RT-PCR assay, they are not reliable and therefore not included in the evaluation of the variability between the different assays. Compared to the results from the *ureA*-based SYBR green RT-PCR, the cq-values from the *H. suis* positive samples obtained by the *carR* probe-based RT-PCR were less variable resulting in a lower cq-mean and standard deviation (Table 3 and Supplementary Table 3). This finding clearly indicates a higher diagnostic specificity and analytical sensitivity for the *carR* probe-based RT-PCR.

In our study, no aspecific reactions with human, non-human primate, murine and porcine DNA nor with DNA from other bacteria could be observed when using the *carR* probe-based RT-PCR. As only relatively low amounts of *H. suis* DNA have been detected in human gastric biopsies using RT-PCR and large amounts of host DNA are present in these samples, the use of the target-specific *carR* probe may be very useful to detect *H. suis* DNA in human gastric biopsies.

2) Optimization of a mouse-passage-based isolation protocol for *Helicobacter suis*

Optimization of a mouse-passage *H. suis* isolation method

Overgrowth with *Lactobacillus* spp. has been reported as one of the main reasons for failure of *H. suis* isolation from the murine and human stomach (Matsui et al., 2014; unpublished data). In order to inhibit the growth of contaminants, including *Lactobacillus* spp., and allow cultivation of *H. suis,* linezolid was added to the biphasic *H. suis* isolation medium. Linezolid is a synthetic oxazolidone, inhibiting the protein synthesis by binding to the 50S ribosomal subunit of the peptidyltransferase center of the bacterial ribosomes. It is active against a broad spectrum of Gram-positive bacteria, including many *Lactobacillus* species (Agar and Gould, 2012), and has a relatively low activity (MIC_{90} 8 µg/ml) against *H. pylori* (Sanchez et al., 2000).

The linezolid-supplemented biphasic growth medium and the *H. suis* growth medium without linezolid (control sample) were inoculated with homogenized gastric tissue samples from mice, infected for 4 weeks with a

dilution series of viable *H. suis.* All plates with growth medium that did not contain linezolid, were overgrown by contaminants, making isolation of *H. suis* impossible. On linezolid-supplemented biphasic growth medium inoculated with stomach homogenate of mice infected with 10^8, 10^7, 10^6, 10^5 and 10^4 bacteria, pure *H. suis* isolates were obtained. *H. suis* bacteria could be observed after 2-3 days of *in vitro* cultivation. On the contrary, after two weeks of incubation, attempts to isolate *H. suis* from the stomach of mice infected with fewer than 10^4 viable bacteria remained unsuccessful, indicating that the minimal infection dose of *H. suis* in mice is at least 10^4 viable *H. suis* bacteria/ml. This finding has also been confirmed in other reports (Matsumoto et al., 2009; Matsui et al., 2014).

Low recombination events and maintenance of integrity in the *H. suis* genome

Whole genome analysis of the 4 isolates obtained after mouse-passage of porcine *H. suis* HS1 strain (HS1_O1, HS1_O2, HS1_O3 and HS1_O4) and the original porcine HS1_I1 isolate, showed that mouse-passage of *H. suis* did not result in major recombination events within the *H. suis* genome (Figure 4). Similar findings were reported for *H. pylori* after mouse-passage (Lundin et al., 2005). This may indicate that *H. suis* strains obtained using this mouse-passage-based isolation protocol can be used to further characterize the agent.

Figure 4

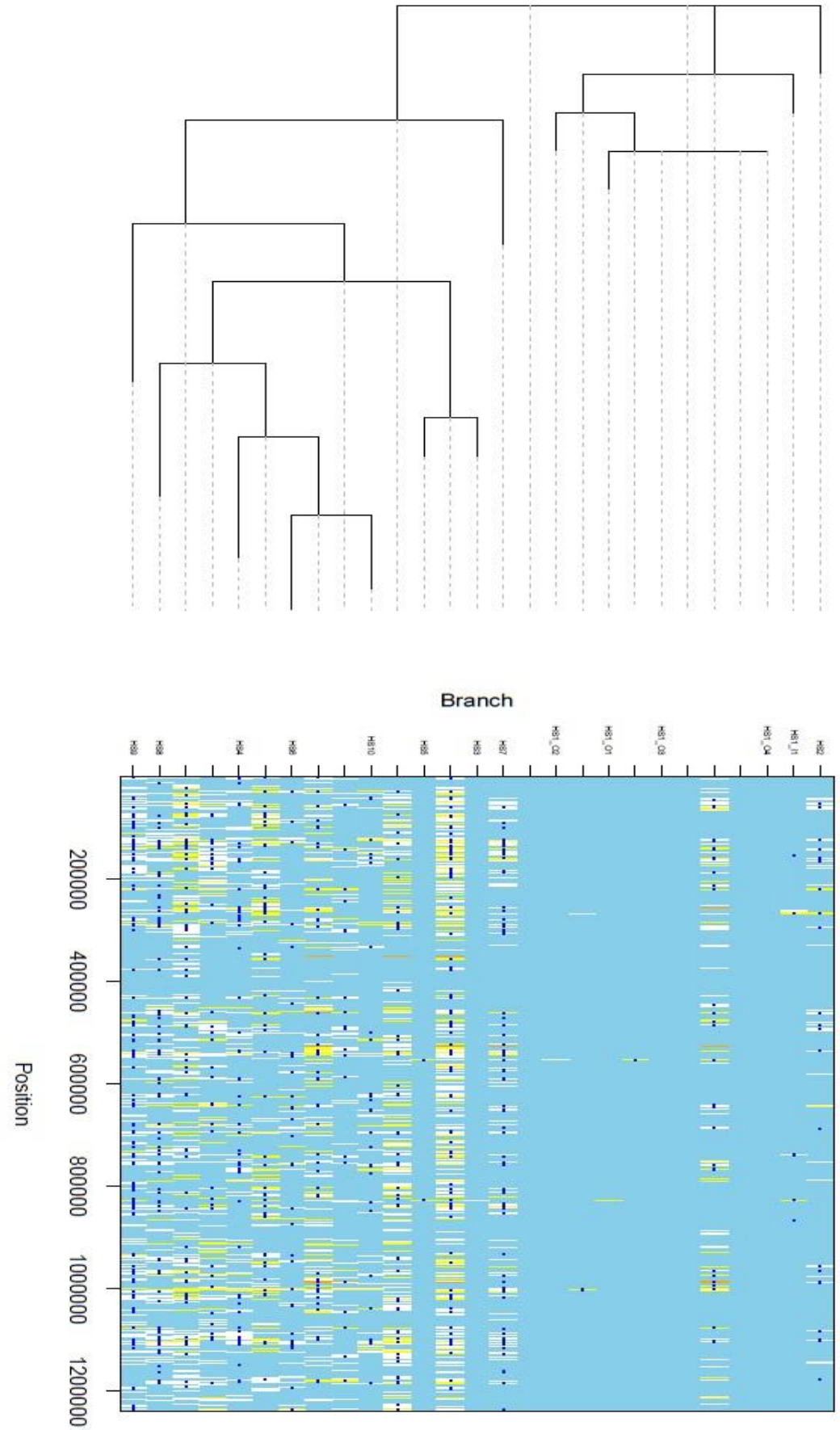
Branch
Position
200000
400000
600000
800000
1000000
1200000

Figure 4: Phylogenetic tree in combination with an overview of the recombination events. Shown are the different recombination events in combination with their position detected after whole genome comparison of the different *H. suis* strains, successively HS2, HS1_I1, HS1_o4, HS1_O3, HS1_O1, HS1_O2, HS7, HS3, HS5, HS10, HS6, HS4, HS8, HS9.

Conclusions

The developed *carR* probe-based RT-PCR appears to be a reliable and accurate diagnostic method that can be applied for the detection and quantitation of *H. suis* in gastric samples. Since no interactions with other NHPH, nor with host DNA could be observed, the use of this technique is recommended for the screening of gastric biopsies, derived from human patients and animals. Using the optimized isolation protocol, we succeeded in isolating relatively low numbers of *H. suis* bacteria from the mouse stomach. Application of this technique may help to obtain and characterize pure human-derived *H. suis* isolates after mouse-passage of *H. suis*-infected human gastric biopsies.

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LEGENDS OF SUPPLEMENTARY TABLES

Supplementary Table 1 Overview of the separate cq-values, means and standard deviations for the standard dilution series within one run using the *carR* probe-based RT-PCR

Supplementary Table 2 Overview of the separate cq-values, means and standard deviations for the standard dilution series between 3 different runs using the *carR* probe-based RT-PCR

Supplementary Table 3 Detailed overview on the *H. suis* status and the obtained cq mean values after analysis of the gastric samples from different hosts with the *ureA*-based SYBR green RT-PCR, the *carR* probe-based RT-PCR and the *carR*-based SYBR green RT-PCR

TABLES

Table 1 Overview of the bacteria used to verify the diagnostic specificity of the probe-based RT-PCR for *H. suis* .

Group	Species	Isolate(s)	Class	Order	Amplification *ureA*-based SYBR green RT-PCR	Amplification *carR* probe-based RT-PCR	Amplification *carR* -based SYBR green RT-PCR
***Helicobacter* spp.**	*H. suis*	HS1-2,HS4-9	Epsilonproteobacteria	Campylobacterales	Yes	Yes	Yes
	H. heilmannii	ASB1.4/11/9.4/7.1/6.3	Epsilonproteobacteria	Campylobacterales	No	No	No
	H. ailurogastricus	ASB21_2/_7/_11	Epsilonproteobacteria	Campylobacterales	Yes	No	No
	H. bizzozeronii	r53/t10/ASB22 kol5/Yryla/m20/ Heydar	Epsilonproteobacteria	Campylobacterales	Yes	No	No
	H. felis	ds1/m26/m29/m38-39/dog7/jkm3/cs5-7/106	Epsilonproteobacteria	Campylobacterales	Yes	No	No
	H. salomonis	m45/Yvira2/alma 0595/Inninen/	Epsilonproteobacteria	Campylobacterales	No	No	No
	H. pylori	Qir3/stm0138/J99/PMSS1/SS1	Epsilonproteobacteria	Campylobacterales	No	No	No
	H. cynogastricus	JKM4	Epsilonproteobacteria	Campylobacterales	Yes	No	No
	H. baculiformis	m50	Epsilonproteobacteria	Campylobacterales	Yes	No	No
	H. cetorum	015903/016202	Epsilonproteobacteria	Campylobacterales	No	No	No
	H. cholecystus	r3555	Epsilonproteobacteria	Campylobacterales	No	No	No
	H. trogontum	r3554	Epsilonproteobacteria	Campylobacterales	No	No	No
	H. acinonychis	Hacino1-3	Epsilonproteobacteria	Campylobacterales	No	No	No
	H. mustellae	img8776	Epsilonproteobacteria	Campylobacterales	No	No	No
	H. pullorum	cb103	Epsilonproteobacteria	Campylobacterales	No	No	No
	H. marmotae	cb55	Epsilonproteobacteria	Campylobacterales	No	No	No
	H. mesocricetorum	ccug45420	Epsilonproteobacteria	Campylobacterales	No	No	No
	H. bilis	atcc49320	Epsilonproteobacteria	Campylobacterales	No	No	No
	H. equorum	EqF1	Epsilonproteobacteria	Campylobacterales	No	No	No
	H. canadensis	H7163	Epsilonproteobacteria	Campylobacterales	No	No	No
***Campylobacter* spp.**	*C. jejuni*	KC40/TB4	Epsilonproteobacteria	Campylobacterales	No	No	No
	C. coli	Grp028	Epsilonproteobacteria	Campylobacterales	No	No	No
	C. upsaliensis	img19529	Epsilonproteobacteria	Campylobacterales	No	No	No
	C. fetus	V24-6	Epsilonproteobacteria	Campylobacterales	No	No	No
Other bacteria	*Lactobacillus reuteri/ L. gasseri*	Cb110/Cb108	Bacilli	Lactobacillales	No	No	No
	Clostridium	LR3869	Clostridia	Clostridiales	No	No	No

	perfringens						
	Staphylococcus aureus/S. pseudintermedius	LR3843/LR3821	Bacilli	Bacillales	No	No	No
	Streptococcus suis/ S. equi subsp. *equi/ S. equi* subsp. *Zooepidemicus*	LR3841/LR3876/ LR3883	Bacilli	Lactobacillales	No	No	No
	Pasteurella multocida	LR3892	Gammaproteobacteria	Pasteurellales	No	No	No
	Mannheimia haemolytica	LR3870	Gammaproteobacteria	Pasteurellales	No	No	No
	Yersinia pseudotuberculosis	LR3798	Gammaproteobacteria	Enterobacteriales	No	No	No
	Pseudomonas aeruginosa	LR3861	Gammaproteobacteria	Pseudomonadales	No	No	No
	Klebsiella pneumoniae	LR3806	Gammaproteobacteria	Enterobacteriales	No	No	No
	Proteus vulgaris	LR1537	Gammaproteobacteria	Enterobacteriales	No	No	No
	Bacillus licheniformis	DMS347	Bacilli	Bacillales	No	No	No
	Salmonella Typhimurium	LR3862	Gammaproteobacteria	Enterobacteriales	No	No	No
	Trueperella pyogenes	LR3863	Actinobacteria	Actinomycetales	No	No	No
	Escherichia coli	LR3889	Gammaproteobacteria	Enterobacteriales	No	No	No
	Aeromonas hydrophila	LR3758	Gammaproteobacteria	Aeromonadales	No	No	No
	Fusobacterium gastrosuis	CDW01	Fusobacteria	Fusobacteriales	No	No	No
	Enterococcus faecalis	LR3751	Bacilli	Lactobacillales	No	No	No
	Actinobacillus equuli	*ccug 19799T*	Gammaproteobacteria	Pasteurellales	No	No	No

Table 2 Overview of the number of positive gastric samples, using the *ureA*-based RT-PCR, the *carR* probe-based RT-PCR and the *carR*-based SYBR green RT-PCR. Gastric samples were derived from pigs (natural host), humans, non-human primates (natural host) and mice (experimental infections).

Host gastric samples	Number of samples	Number of *H. suis* positive samples	Number of positive samples using *ureA*-based RT-PCR	Number of positive samples using *carR* probe-based RT-PCR	Number of positive samples using *carR*-based SYBR green RT-PCR
Human (with or without natural *H. suis* infection)	80	6	7	6	0
Non-human primates (with or without natural *H. suis* infection)	9	8	9	8	6
Mice (experimentally infected animals and control animals)	8	4	4	4	0
Pigs (with or without natural *H. suis* infection)	9	5	5	5	0

Table 3 Overview of the mean number and standard deviation of *H. suis* bacteria/µl (cq-mean) in the positive gastric samples per host, using *ureA*-based RT-PCR and *carR* probe-based RT-PCR respectively. Gastric samples were derived from pigs, humans, non-human primates and mice.

	Cq-mean *ureA*-based SYBR green RT-PCR	Cq-mean *carR* probe-based RT-PCR	Stdev *ureA*-based SYBR green RT-PCR	Stdev *carR* probe-based RT-PCR
Human positive gastric samples	9.68E+02	2.68E+01	2.13E+03	3.49E+01
Non-human primate positive gastric samples	1.38E+06	7.79E+04	3.95E+06	1.55E+05
Murine positive gastric samples	1.14E+04	4.20E+03	3.85E+03	2.41E+03
Porcine positive gastric samples	5.82E+02	2.50E+02	7.94E+02	3.44E+02

SUPPLEMENTARY TABLES

Supplementary Table 1

	1,00E+00	1,00E+01	1,00E+02	1,00E+03	1,00E+04	1,00E+05	1,00E+06	1,00E+07	1,00E+08	1,00E+09
Standard 1	37.57	33.47	29.07	25.38	21.98	18.49	15.16	11.92	7.53	3.26
Standard 2	35.03	32.32	29.1	25.92	22.11	18.99	15.21	11.86	7.93	3.59
Standard 3	36.49	33.11	29.08	26.03	21.93	18.42	15.25	11.98	7.67	3.08
Standard 4	35.86	32.59	29.28	25.52	22.01	18.98	15.1	11.84	7.52	3.48
Standard 5	36.99	32.75	28.85	25.5	22.05	18.74	15.04	11.75	7.68	3.57
Standard 6	35.37	33.03	29.31	25.58	22.09	18.96	15.43	11.85	7.9	3.56
Mean	36.2183	32.878	29.115	25.655	22.028	18.7633	15.1983	11.8667	7.705	3.42333
SD	0.97534	0.4094	0.1667	0.25859	0.0682	0.25696	0.13615	0.07789	0.17627	0.20772
Efficiency run (%)	89.9									
Linear correlation (r^2)	0.998									

Supplementary Table 2

	1,00E+00	1,00E+01	1,00E+02	1,00E+03	1,00E+04	1,00E+05	1,00E+06	1,00E+07	1,00E+08	1,00E+09
Run 1	37.07	32.43	28.48	25.09	21.58	18.25	14.92	11.57	6.99	2.47
	36.41	31.98	28.42	24.96	21.59	18.28	14.93	11.73	7.11	1.79
	36.39	31.66	28.46	25.07	21.56	18.28	14.86	11.46	7.19	2.35
Run 2	34.66	31.71	28.47	25.15	21.92	18.43	14.8	11.67	6.96	2.97
	35.51	32.6	28.54	25.18	21.83	18.45	14.92	11.76	7.76	3.44
	36.03	32	28.62	25.17	21.82	18.41	14.93	11.65	7.29	3.07
Run 3	no value (nv)	32.37	29.18	26.11	22.25	18.6	15.57	12.09	7.83	3.24
	36.14	32.62	29.3	25.99	22.37	18.66	15.62	12.13	8.01	3.8
	nv	32.78	29.26	25.55	22.24	18.61	15.53	11.97	7.8	3.24
Mean	36.03	32.239	28.7478	25.3633	21.907	18.4411	15.12	11.7811	7.43778	2.93
SD	0.7654	0.4125	0.3796	0.42205	0.3131	0.15463	0.34329	0.23267	0.40862	0.62028
Mean efficiency run 1,2 and 3	90.03									
Mean linear correlation run 1,2 and 3	0.998									

Supplementary Table 3

Samples	*H. suis* status	*UreA*-based SYBR green RT-PCR	*CarR* probe-based RT-PCR	*CarR*-based SYBR green RT-PCR
Preserve Human DNA (n=80)				
human 1	Absent	Negative	Negative	Negative
human 2	Absent	Negative	Negative	Negative
human 3	Absent	Negative	Negative	Negative
human 4	Absent	Negative	Negative	Negative
human 5	Absent	Negative	Negative	Negative
human 6	Absent	Negative	Negative	Negative
human 7	Absent	Negative	Negative	Negative
human 8	Absent	Negative	Negative	Negative
human 9	Absent	Negative	Negative	Negative
human 10	Absent	Negative	Negative	Negative
human 11	Absent	Negative	Negative	Negative
human 12	Absent	Negative	Negative	Negative
human 13	Absent	Negative	Negative	Negative
human 14	Absent	Negative	Negative	Negative
human 15	Absent	Negative	Negative	Negative
human 16	Absent	Negative	Negative	Negative
human 17	Absent	Negative	Negative	Negative
human 18	Absent	Negative	Negative	Negative
human 19	Absent	Negative	Negative	Negative
human 20	Absent	Negative	Negative	Negative
human 21	Absent	Negative	Negative	Negative
human 22	Absent	Negative	Negative	Negative
human 23	Absent	Negative	Negative	Negative
human 24	Absent	Negative	Negative	Negative
human 25	Absent	Negative	Negative	Negative
human 26	Absent	Negative	Negative	Negative
human 27	Absent	Negative	Negative	Negative
human 28	Absent	Negative	Negative	Negative
human 29	Absent	Negative	Negative	Negative
human 30	Absent	Negative	Negative	Negative
human 31	Absent	Negative	Negative	Negative
human 32	Absent	Negative	Negative	Negative
human 33	Absent	Negative	Negative	Negative
human 34	Absent	Negative	Negative	Negative

human 35	Absent	Negative	Negative	Negative
human 36	Absent	Negative	Negative	Negative
human 37	Absent	Negative	Negative	Negative
human 38	Absent	Negative	Negative	Negative
human 39	Absent	Negative	Negative	Negative
human 40	Absent	Negative	Negative	Negative
human 41	Absent	Negative	Negative	Negative
human 42	Absent	Negative	Negative	Negative
human 43	Absent	Negative	Negative	Negative
human 44	Absent	Negative	Negative	Negative
human 45	Absent	Negative	Negative	Negative
human 46	Absent	Negative	Negative	Negative
human 47	Absent	Negative	Negative	Negative
human 48	Absent	Negative	Negative	Negative
human 49	Absent	Negative	Negative	Negative
human 50	Absent	Negative	Negative	Negative
human 51	Absent	Negative	Negative	Negative
human 52	Absent	Negative	Negative	Negative
human 53	Absent	Negative	Negative	Negative
human 54	Absent	Negative	Negative	Negative
human 55	Absent	Negative	Negative	Negative
human 56	Absent	Negative	Negative	Negative
human 57	Absent	Negative	Negative	Negative
human 58	Absent	Negative	Negative	Negative
human 59	Absent	Negative	Negative	Negative
human 60	Absent	Negative	Negative	Negative
human 61	Absent	Negative	Negative	Negative
human 62	Absent	Negative	Negative	Negative
human 63	Absent	Negative	Negative	Negative
human 64	Absent	Negative	Negative	Negative
human 65	Absent	Negative	Negative	Negative
human 66	Absent	Negative	Negative	Negative
human 67	Absent	Negative	Negative	Negative
human 68	Absent	Negative	Negative	Negative
human 69	Absent	Negative	Negative	Negative
human 70	Absent	Negative	Negative	Negative
human 71	Absent	Negative	Negative	Negative
human 72	Absent	Negative	Negative	Negative
human 73	Absent	Negative	Negative	Negative
human 74	Present	2.51E+01	2.05E+00	Negative
human 75	Present	1.07E+02	3.39E+01	Negative

human 76	Present	5.78E+03	9.21E+01	Negative
human 77	Present	4.65E+02	1.74E+00	Negative
human 78	Absent	2.12E+01	Negative	Negative
human 79	Present	3.32E+02	2.74E+01	Negative
human 80	Present	4.39E+01	3.69E+00	Negative
Non-human primate DNA (n=9)				
JI 503 biopt a	Present	1.49E+02	4.96E+00	Negative
Ro2019 bio a	Present	2.89E+04	2.71E+03	7.14E+02
r08041 bio a	Present	1.74E+04	1.28E+04	Negative
JI 190 bio a	Absent	3.96E+02	Negative	Negative
HSM1Ro40522 dna	Present	1.19E+07	4.54E+05	7.60E+05
baviaan 9 bio a	Present	1.03E+05	9.08E+04	2.02E+03
baviaan 9 bio f	Present	2.34E+04	1.34E+04	1.08E+03
rhesus aap bio a	Present	9.36E+04	1.92E+04	2.72E+03
rhesus aap bio f	Present	2.53E+05	3.04E+04	4.79E+03
Mouse DNA (n=8)				
1m2 c1 c	Absent	Negative	Negative	Negative
1m2 c2 c	Absent	Negative	Negative	Negative
1m2 c3 c	Absent	Negative	Negative	Negative
1m2 c4 c	Absent	Negative	Negative	Negative
1m2 s1 c	Present	1.22E+04	3.66E+03	Negative
1m2 s2 c	Present	1.60E+04	7.61E+03	Negative
1m2 s3 c	Present	1.08E+04	3.63E+03	Negative
1m2 s4 c	Present	6.68E+03	1.91E+03	Negative
Pig DNA (n=9)				
p17 c	Absent	Negative	Negative	Negative
p18 c	Absent	Negative	Negative	Negative
p20 c	Absent	Negative	Negative	Negative
p11 c	Present	1.72E+03	5.14E+02	Negative
p27 c	Present	4.03E+01	8.42E+00	Negative
p28 c	Present	2.22E+01	5.05E+00	Negative
p33 c	Absent	Negative	Negative	Negative
p49 c	Present	7.15E+00	2.25E+00	Negative
p50 c	Present	1.12E+03	7.22E+02	Negative

GENERAL DISCUSSION

..."all the diseases begin in the gut"... by Hippocrates

Besides the well-known human pathogen *Helicobacter pylori* (*H. pylori*), non-*Helicobacter pylori Helicobacter* (NHPH) species from domestic animals have also been associated with gastric disease in humans. Many of these very fastidious bacteria have recently been *in vitro* isolated from their natural hosts, which has led to progress in understanding the pathogenesis of NHPH-related pathologies. A distinct feature is their association with gastric mucosa-associated lymphoid tissue (MALT) lymphoma in humans, but this is most probably a tip of the iceberg…

Increased frequency of gastric *H. suis* infections in human patients with Parkinson's disease (PD): coincidence or an underlying cause in the pathogenesis of this disease?

H. pylori may interfere with many biological processes, both inside and outside the stomach, possibly influencing or determining the occurrence and/or outcome of many extragastric diseases (Alvarez-Arellano et al., 2014; Budzynski et al., 2014; Smyk et al., 2014; Testerman et al., 2014; Wong et al., 2014). While its role in idiopathic thrombocytopenic purpura (Radic et al., 2014; Hasni et al., 2011; Yamanishi et al., 2006) and sideropenic anemia (Kang et al., 2011; Monzon et al., 2013) has already been acknowledged, emerging evidence suggests that *H. pylori* may be associated with neurodegenerative disorders as well (Csoti et al., 2016; Alvarez-Arellano et al., 2014; Budzynski et al., 2014; Dobbs et al., 2008; Dobbs et al., 2010; Dobbs et al., 2016; Nielsen et al., 2012; Rees et al., 2011). PD is a degenerative disorder of the central nervous system (CNS) characterized by cell death of dopamine-generating neurons in the substantia nigra, a region of the basal ganglia of the brain (Wirdefeldt et al., 2011; Mhyre et al., 2012; Kannarkat et al., 2013). The familial form of PD is caused by mutations in several different genes (including the *α-synuclein* gene and the *parkin* gene), whereas the aetiology of non-heritable idiopathic Parkinsonism (IP) remains unknown, although several risk factors, such as rural residence, livestock farming, head trauma, well-water drinking, infectious diseases and exposure to pesticides, have been associated with the appearance of this disease state (Lee et al., 2002; Schiesling et al, 2008, Dobbs et al., 2008; Hardy et al., 2006). Besides aberrations in motor functions, increased gastrointestinal dysfunction is well-acknowledged in patients with parkinsonism and usually precedes the diagnosis of PD by many years (Edwards et al., 1992; Charlett et al., 1997; Abbott et al., 2001; Pfeiffer, 2011; Dobbs et al., 2016). The finding of prodromal peptic ulcers in patients with PD in 1965 (Strang, 1965) paved the way to explore any link between (the then not yet described (Warren et al, 1983)) *H. pylori* and PD. Infection with *H. pylori* has been shown to be an arbiter for progression of the poverty of movement (hypokinesia) (Dobbs et al., 2008; Dobbs et al., 2010; Dobbs et al., 2012; Dobbs et al., 2013).

In some cases, decreased absorption of levodopa, which is the main drug used to treat PD, may be involved (Pierantozzi et al., 2001; Hashim et al., 2014). Indeed, eradicating *H. pylori* in PD patients by applying antibiotic treatment results in an increased absorption of levodopa and decreased motor fluctuations (Hashim et al., 2014). Although it remains unknown how *H. pylori* may interfere with levodopa absorption, several hypotheses have been proposed. Since the solubility of levodopa is pH dependent, *H. pylori*-induced gastric pH alterations

might influence absorption (Annibale et al., 2002). Furthermore, levodopa absorption may also be altered by *H. pylori*-associated gastric myoelectric dysfunctioning resulting in delayed gastric emptying (Miyaji et al., 1999). Finally, it has been suggested that *H. pylori* might utilize levodopa for growth (Doherty et al., 2009).

Whilst influence of *H. pylori* on levodopa absorption may be relevant to symptomatic treatment of PD, it does not provide evidence on aetio-pathogenesis. This was shown in a randomized, double-blind, placebo-controlled trial. Objectively-measured gait hypokinesia improved over the year following biopsy-proven (culture and polymerase chain reaction (PCR)) *H. pylori* eradication, and did not deteriorate over the next two years. The effect on hypokinesia was seen in patients never given symptomatic treatment for PD, and was no greater in those receiving background anti-parkinsonian medication. Thus, it was not due to improved absorption of parkinsonian medication (Dobbs et al 2010). This was confirmed in a longitudinal observational study of the effects of routine antimicrobial use on PD. Hypokinesia again improved after treating *H. pylori* infection, but antimicrobials given for other indications did not affect it (Dobbs et al 2013).

Activation of microglia cells, resulting in neuroinflammation and apoptosis of nerve cells as a consequence of chronic gastric inflammation may be a possible explanation for the association that was found between *H. pylori* infection and neurodegenerative disorders (Dobbs et al., 2000; Dobbs et al., 2008; Dobbs et al., 2010; Dobbs et al., 2012; Kountouras et al., 2012; Dobbs et al., 2016). This is described in more detail in the next heading, dealing with the stomach-brain axis.

Molecular mimicry between *H. pylori* antigens and human antigens, presented by dopamine producing neurons has also been proposed (Dobbs et al., 2000; Schulz et al., 2006; Dobbs et al., 2008; Dobbs et al., 2010; Dobbs et al., 2012; Dobbs et al., 2016). There is molecular mimicry between *H. pylori* and gastric antigens (Appelmelk et al., 1997) and other studies reported the presence of auto-antibodies against dopamine-producing neurons in blood and the cerebrospinal fluid in patients with PD (Poupard and Emilie, 1984; Fiszer et al., 1996; Schulz et al., 2006; Dobbs et al., 2008; Dobbs et al., 2010; Dobbs et al., 2012; Dobbs et al., 2016).

It has been suggested that living or working on livestock farms may increase the risk of PD (Gorell et al., 1998; Lee et al., 2002; Wirdefeldt et al., 2011), raising the possibility that zoonotic transmission of animal-associated gastric helicobacters might also contribute to the etiology of this disease. There has been much attention to the possible dangers of herbicides, but arable farmers have a reduced risk of dying from PD (Lee et al., 2002). *H. suis* is highly prevalent in the stomach of pigs and is the most prevalent NHPH species in humans (Haesebrouck et al., 2009). A case report published in 2013 described a *H. suis* infection in a pig veterinarian suffering from reflux esophagitis and general dyspeptic symptoms (Joosten et al., 2013). Using multilocus sequence typing (MLST), it was demonstrated that the *H. suis* strain, detected in the stomach of the pig veterinarian, was closely related to porcine strains confirming the zoonotic potential of this *Helicobacter* species (Liang et al., 2013). It has been suggested that direct contact with pigs might be an important source for *H. suis* infection in humans (Haesebrouck et al., 2009; De Groote et al., 2005; Van den Bulck et al., 2005; Baele et al., 2008). Additionally, the consumption of raw or undercooked pork might also constitute a source of *H. suis* infection in humans (De Cooman et al., 2013).

Only few information about a possible association between gastric *H. suis* infections and PD was available at the onset of this thesis. There was a report of improvement in PD following eradiation of a spiral gastric NHPH, reported at that time to be *H. heilmannii* (Dobbs et al., 2005), but which we showed to be *H. suis*. Therefore, **in the first chapter** of this thesis, we reinvestigated deoxyribonucleic acid (DNA), extracted from human gastric biopsy samples and archived at a *Helicobacter* reference laboratory, for the presence of *H. suis*. An exceptional high frequency (27%) of *H. suis* DNA was found in extracts from patients with PD, compared with those from a routine gastroenterology service referrals, in whom no PD had been documented (2%). *H. suis* DNA was more often detected in patients with biopsy proven *H. pylori* eradication. Based on this finding, it can be hypothesized that these patients were co-infected with *H. pylori* and *H. suis* as described before (Liu et al., 2015). Triple therapy, usually consisting of amoxicillin, clarithromycin and a proton pump inhibitor, may have been ineffective to eradicate *H. suis* giving this bacterium the opportunity to multiply in the human stomach and to cause gastric disease. Results of previous studies gave evidence of intrinsic reduced susceptibility to aminopenicillins of porcine *H. suis* isolates compared to *H. pylori* (Vermoote et al., 2011). In addition, recent *in vitro* antimicrobial susceptibility testing showed evidence of acquired resistance to clarithromycin among *H. suis* isolates (De Witte Chloë, 2017, personal communication). Both findings strengthen the above mentioned assumption. Indeed, in the case referred to above (Dobbs et al 2005), there was short-lived improvement in PD following standard triple therapy, which relapsed with recrudescence of *H. suis*. Successful (biopsy-proven) *H. suis* eradication resulted in long-term improvement. Nevertheless, successful eradication of *H. suis* has been described after treatment with amoxicillin, clarithromycin and a proton pump inhibitor (Joosten et al., 2013; Hellemans et al., 2005). These differences in treatment outcome, might support previous suggestions on the occurrence of acquired antimicrobial resistance (Vermoote et al., 2011; De Witte et al., 2017, personal communications).

We additionally described the presence of *H. suis* DNA in a blood sample of a patient with PD and unpublished results from recent *in vivo* experiments in mice showed the presence of *Helicobacter* antigens in macrophages at the level of mesenteric lymph nodes during chronic infection. Together, all these findings suggest that the effects caused by *H. suis* are not restricted to the stomach. Moreover, *H. pylori* DNA, and even culturable organisms have been found in gastric lymph nodes from human surgical specimens (Ito et al., 2008). Clearly, the role of *Helicobacter* in general and *H. suis* in particular, in PD requires further investigation.

The stomach-brain axis in relation to a *H. suis* infection: does *H. suis* affect neuroinflammation?

Evidence is accumulating that neuroinflammation plays an important role in the pathophysiology of PD. Activated microglia can be detected in the brain of PD patients, reflected by a change in morphology from ramified to a more round shape (Wang et al., 2015; Tansey et al., 2007; Hirsch and Hunot, 2009). Microglia activation results in the release of pro-inflammatory cytokines which can act on neuronal integrity (Wang et al., 2015; Fonseca et al., 2014). The intermediate stage, “primed microglia”, describes the atypical microglial stage, which precedes a further neurotoxic microglial activation as a consequence of a secondary pro-inflammatory stimulus which can come from the periphery (Perry et al., 2013). Indeed, peripheral inflammation can activate microglia, in particular when the brain barriers are disturbed (Wang et al., 2015). Several specialized CNS

barriers, such as the blood-brain barrier (BBB) and the blood-cerebrospinal fluid (CSF) barrier, play a crucial role in the maintenance of brain homeostasis, since they determine what will (and what will not) enter the brain (De Bock et al., 2014). The BBB, formed by capillary endothelial cells which are connected by tight junctions, separates the circulating blood from the extracellular fluid in the CNS. The blood-CSF barrier is established by the choroid plexus epithelial cells, which secrete the CSF and have an essential function in the communication between the peripheral immune system and the CNS (De Bock et al., 2014). Dysfunction of these brain barriers has been implicated in the pathogenesis of PD (Vawter et al., 1996; Pisani et al., 2012; Stolp et al., 2009; Lee et al., 2014).

Interactions between the gastrointestinal tract and the brain are well recognized (Cryan et al., 2012) and in particular, a bidirectional communication between the CNS and the gastrointestinal tract via the gut-brain axis has been considered. Moreover, it has been suggested that neuroinflammation could emanate from infection in the gastrointestinal tract (Dobbs et al., 2000; Dobbs et al., 2008; Dobbs et al., 2010; Ferrari et al., 2011; Dobbs et al., 2013; Dobbs et al., 2016). There is evidence that intestinal infection or dysbiosis plays a part in the pathogenesis. There are biological gradients between leukocyte subset counts and facets on PD (Dobbs et al 2012). The same subsets are associated with hydrogen-breath-test positivity for small-intestinal bacterial overgrowth. In PD, successive courses of antimicrobials are associated with cumulative increase in flexor rigidity (Dobbs et al. 2013), which may suggest progressively increasing colonic dysbiosis (Carethers, 2011). There still appears to be a specific effect of *Helicobacter*: it had an effect on hypokinesia in addition to that on natural-killer cell count (Dobbs et al 2012), and routine treatment of *Helicobacter* produced an indication specific improvement in hypokinesia (Dobbs et al 2013). Gastric *Helicobacter* spp. generally cause a life-long gastric infection since the induced host immune response is not able to eradicate the infection, resulting in persistent inflammation and gastric epithelial cell damage (Montecucco & Rapuoli, 2001; Flahou et al., 2011; Flahou et al., 2012).

A recently proposed hypothesis is summarized in **Figure 1**: *Helicobacter* might influence the brain through the blood, since injury of the gastric mucosa might lead to leakage of bacterial components and metabolites, and even pro-inflammatory factors into the blood circulation resulting in systemic inflammation (Fukuda et al., 2001; Sun et al., 2004; Fedwick et al., 2005; Guttman and Finlay, 2009; Wroblewski et al, 2011; Caron et al., 2015; Cryan et al., 2014; Mayer et al., 2014). These inflammatory mediators may then contribute to disturbance of the brain homeostasis leading to neurodegenerative diseases (Dobbs et al., 2000; Dobbs et al., 2008; Dobbs et al., 2010; Dobbs et al., 2012; Kontouras et al., 2015; Dobbs et al., 2016; Charlett et al. 1998; Dobbs et al. 1999; Reale et al, 2009). Furthermore, several reports suggested the involvement of systemic inflammation in the impairment of the BBB (Kortekaas et al., 2005; Stolp et al., 2009) and the blood-CSF barrier (Vandenbroucke et al., 2012; Demeestere et al., 2015; Villaran et al., 2010; Perry et al., 2013).

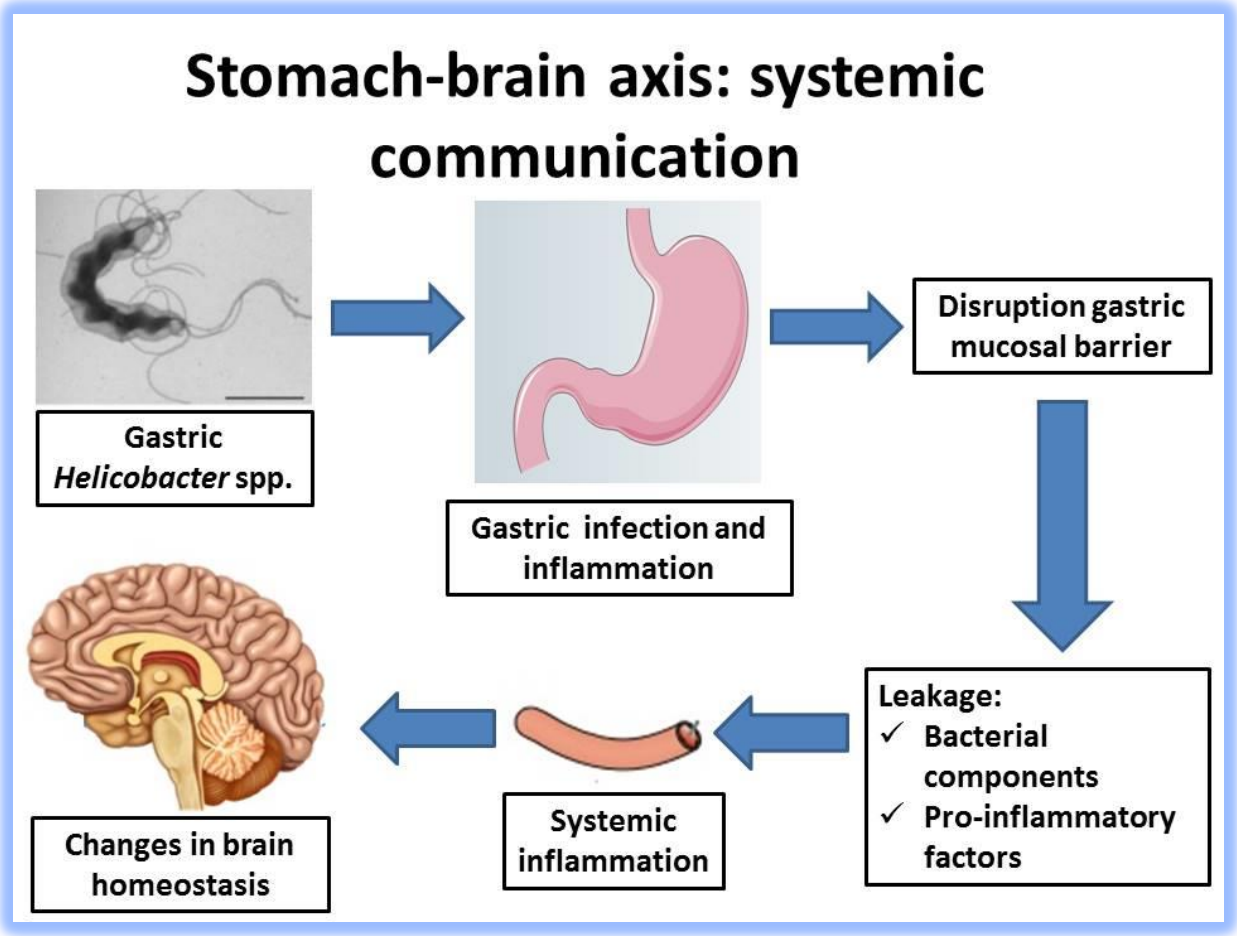

Figure 1: Stomach-brain axis via systemic circulation. A gastric infection with *Helicobacter* may evoke inflammation resulting in a disruption of the gastric mucosal barrier. In turn this could lead to leakage of bacterial components and pro-inflammatory factors from the stomach into the systemic circulation, resulting in systemic inflammation. These inflammatory mediators may then contribute to disturbance of the brain homeostasis leading to neurodegenerative diseases. Figure was adapted from Baele et al., 2008 (*H. suis),* Cryan et al., 2012 (vessel), Mayer et al., 2014 (brain), Servier Medical Art (stomach).

Elaborating on the high frequency of *H. suis* infections in patients with PD as shown in **chapter 1** and keeping in mind the above mentioned hypothesis that *Helicobacter* might influence the brain through the blood, in the **second chapter** of this thesis we examined whether a gastric *H. suis* infection has an impact on the integrity of the gastric mucosal barrier and the brain barriers through the induction of inflammation. For this purpose, mice were infected with a virulent *H. suis* strain as shown before (Flahou et al., 2010). At 4 weeks post-infection, the inflammatory response induced by *H. suis* was characterized by an infiltration of immune cells in the gastric mucosa and submucosa as well as by the secretion of interleukin (IL)-1β, IL-17 and the human IL-8-related murine cytokines, lipopolysaccharide (LPS)-induced CXC chemokine (LIX) and keratinocyte-derived chemokine (KC), at the site of infection. These findings were in line with previous *H. suis* experimental infection studies as well as with *in vivo* trials with other gastric helicobacters (Flahou et al., 2012; Flahou et al., 2010; Bosschem et al., 2016; Peek et al., 2010; Alvarez-Arellano et al., 2014; Hashim et al., 2014). Since the pro-inflammatory cytokine IL-1β is a potent inhibitor of gastric acid secretion, it facilitates *Helicobacter* colonization of the gastric mucosa (Joosten et al., 2013; Semper et al., 2014). It also plays a crucial role in the development of gastric

pathologies (Joosten et al., 2013). Its secretion depends on the activation of the inflammasome, an intracellular multiprotein oligomer implicated in the activation of inflammatory processes (Semper et al., 2014). *H. pylori* induced inflammasome activation in dendritic cells (DCs) requires the nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing 3 (NLRP3) (Semper et al., 2014) and depends on the presence of the vacuolating cytotoxin (VacA) and cag pathogenicity island (cagPAI) virulence factors (Semper et al., 2014; Guo et al., 2015). In contrast to *H. pylori, H. suis* lacks both VacA and cagPAI (Vermoote et al., 2011). It is currently unknown how this bacterium triggers secretion of IL-1β. Screening of a *H. suis* transposon mutant library, as previously developed for *H. pylori* (Odenbreit et al., 1996), for its ability to activate the inflammasome may help to identify the virulence factors involved in this process.

Besides IL-1β, expression of the IL-8-related murine pro-inflammatory cytokines, LIX and KC were also significantly increased in the gastric mucosa exposed to *H. suis*. IL-8 is an important chemotactic and activating factor for neutrophils (Crabtree and Lindley, 1994) and can be upregulated by IL-1β and tumour necrosis factor (TNF) through the nuclear factor (NF)-κB signalling pathway (Liu et al., 2016; Crabtree and Lindley, 1994; Kim et al., 2001; Torok et al., 2005). Additionally, IL-8 secretion can be stimulated by the presence of IL-17 via the extracellular signal-regulated kinase (ERK) 1/2 mitogen-activating protein (MAP) kinase pathway (Kabir, 2011; Bagheri et al., 2015). The *H. pylori* virulence factor cagPAI and several *H. pylori* outer membrane proteins (OMPs) acting as adhesins (*e.g.* blood group antigen binding adhesion A (BabA), sialic acid binding adhesion A (SabA), outer inflammatory protein A (oipA), etc.), have been shown to be involved in the increased IL-8 production by gastric epithelial cells (White et al., 2015). Both cagPAI and these *H. pylori* adhesins are absent in *H. suis* (Vermoote et al., 2011). Recently, the *Helicobacter* outer membrane protein family (Hof)E and HofF proteins of *H. heilmannii* have been shown to be partly involved in the induction of IL-8 expression in the gastric epithelium (Liu et al., 2016). These OMPs are also present in *H. suis* and it remains to be elucidated whether they are involved in this process as well.

The extensive secretion of IL-8 or the related murine cytokines, LIX and KC, by gastric epithelial cells upon *Helicobacter* infection is probably a key factor in host defenses at the mucosal site. Since the immune mechanisms usually fail to eradicate *H. suis*, resulting in chronic infection (Flahou et al., 2010), continued upregulation of these pro-inflammatory cytokines could lead to gastric mucosal damage (Crabtree and Lindley, 1994). Indeed, in our *in vivo* experiment, the *H. suis*-evoked gastric immune response was accompanied by a disruption of the gastric mucosal barrier. This was characterized by an increased gastrointestinal barrier permeability and alterations in gastric tight junction proteins. A disruption of the gastric epithelial barrier, characterized by a loss of claudin-4, claudin-5 and occludin (Fedwick et al., 2005; Wroblewski et al., 2011) in combination with a reorganization of ZO-1 and claudin-1 (Guttman et al., 2009; Caron et al., 2015) has also been described during *H. pylori* infections.

Tight junctions regulate cell polarity and their formation is dependent on the pseudo-autosomal region (PAR)3-PAR6-atypical protein kinase C (aPKC) (PAR) complex (Vermeer et al., 2003). Loss of tight junctions can be mediated by receptor tyrosine kinases, which are members of the epidermal growth factor receptor (ErbB) family. Activation of ErbB2 has been shown to disrupt polarity by associating with PAR6 and aPKC and blocking the interaction with PAR3 (Aranda et al., 2006). Transmembrane mucins, including mucin MUC13, can

participate in receptor tyrosine kinase activation and can thereby contribute to the disruption of the PAR complex (Ren et al., 2006). Since MUC13 is aberrantly expressed upon *H. suis* infection and inflammation (Liu et al., 2016), it remains to be elucidated whether MUC13 plays a role in gastric mucosal barrier disruption. In order to determine whether MUC13 interacts with one of the members of the ErbB family present on the gastric epithelial cells, co-immunoprecipitation can be performed. Additionally, the role of the MUC13 signaling pathway in the PAR complex and the disruption of tight junctions in the stomach can be investigated using *in vitro* transfection assays. If the transfection experiments indicate that MUC13 plays a role in the tight junction regulation, it is recommended to evaluate whether these changes are correlated with a loss of barrier functionality using an electrical cell impedance system (ECIS) or transepithelial electrical resistance (TEER) measurements in transwells.

Apart from the γ-glutamyl transpeptidase (GGT) enzyme and urease (Flahou et al., 2011), no other information is currently available on *H. suis* virulence factors involved in gastric mucosal damage. Unpublished results from a whole transcriptome analysis of *H. heilmannii* showed that GGT is significantly upregulated when this bacterium is attached to gastric epithelial cells. Other virulence factors which are altered in expression in relation to adherence can thus be potential candidates involved in gastric mucosal barrier disruption and should be further investigated. This investigation can be facilitated by chromosomal insertional mutagenesis approaches and *in vitro* and *in vivo* infection assays using the developed mutants.

Disruption of the gastric mucosal barrier may result in leakage of pro-inflammatory cytokines, chemokines and bacterial metabolites and components, such as LPS, into the blood circulation, thereby inducing systemic inflammation (Cryan et al., 2012; Mayer et al., 2014). In our study, significantly increased levels of IL-1β and the IL-8-related murine cytokine, macrophage inflammatory protein (MIP)1α, were found in the serum of *H. suis*-infected mice resulting in systemic inflammation. This might thus lead to brain barrier dysfunction and subsequent alterations in brain homeostasis (Alvarez-Arellano et al., 2014; Mulak et al., 2015), as might the elevated serum cortisol and IL-6 found in PD (Charlett et al., 1998; Dobbs et al., 1999). Indeed, the integrity of the brain barriers in the *H. suis*-infected mice was compromised. Infection with *H. suis* resulted in changes in tight junction expression of the BBB and the blood-CSF barrier, but increased permeability was only detected at the blood-CSF barrier of *H. suis*-infected animals. Also neuroinflammation, characterized by the activation of microglia cells and the upregulation of pro-inflammatory cytokines, in particular IL-1β and inducible nitric oxide synthase (iNos) in the choroid plexus and IL-6 in the hippocampus of the brain, was detected. Downregulation of tight junction genes in the hippocampus did not result in increased BBB leakage, suggesting that *H. suis* infection has a higher impact on the blood-CSF barrier compared to the BBB. Intraperitoneal injection of mice with LPS, a component which is also present in *H. suis* and other Gram-negative bacteria, also resulted in increased leakage of the blood-CSF barrier as shown before (Vandenbroucke et al., 2012).

Up to date, it remains unknown whether *H. suis* LPS might be involved in the disruption of the integrity of the brain barriers and brain homeostasis. The administration of the antibiotic polymyxin B, which inhibits different biological activities induced by LPS, might be useful to answer this question (Cavaillon et al., 1986). Another possibility to investigate the role of *H. suis* LPS is by creating an LPS mutant of *H. suis,* as already described for *Escherichia coli* (Liu et al., 2015), and repeat the experiment using the LPS-mutant of *H. suis.*

In our studies, fluorescein isothiocyanate (FITC)-dextran was used to determine the BBB and blood-CSF barrier permeability. The application of FICT-dextran is considered a valuable method for the characterization of alterations in permeability of both the BBB and the blood-CSF barrier (Hoffmann et al., 2011; Vandenbroucke et al., 2012). The advantages of the use of FITC dextran as a tool is that FITC dextrans can be obtained in different molecular sizes and that they can be used to determine both solute and ion permeability (small molecular size) and protein permeability (high molecular size) (Hoffmann et al., 2011). However, it is not recommended to use samples from FITC treated animals in combination with paraformaldehyde (PFA) for histological examination, because FITC dextran dissolves in PFA, leading to false negative results (Hoffmann et al., 2011).

An important finding throughout our study was the presence of the pro-inflammatory cytokine IL-1β not only in the inflamed stomach but also in the serum and choroid plexus of the *H. suis*-infected animals, highlighting its role in the induction of neuroinflammation via systemic communication. The role of IL-1β in the integrity of the stomach-brain axis and induction of neuroinflammation requires further investigation. The usage of *H. suis*-infected IL-1β$^{-/-}$ mice might shed more light on this.

Besides systemic communication, neural communication via neuropeptides, such as VIP, Substance P (SP), calcitonin-gene related peptide (CGRP), somatostatin and neuropeptide Y, may also play a role (Delgado et al., 2004; Erin et al., 2012; Mayer et al., 2014; De Vadder et al., 2015). Therefore we have also investigated the effect of an *H. suis* infection on VIP signaling. A remarkable finding in the stomach was the altered expression of the vasoactive intestinal peptide (*VIP*) and its receptor *VIPR1* in *H. suis*-infected animals compared to controls. Neuropeptides, such as VIP, have been shown to promote homeostasis of the mucosal barrier of the gastrointestinal tract and have thus been implicated in tight junction regulation (Chen et al., 2015; Anlauf et al., 2003; Mulak et al., 2015). Alterations in mucosal VIP levels have also been reported in biopsies derived from human patients with chronic gastritis and ulcers (Erin et al., 2012; Chen et al., 2015; Islek et al., 2016; Li et al., 2009; Bercik et al., 2002). Changes in VIP expression might thus induce dysfunction of the gastrointestinal barrier and in this way contribute to the formation of gastric pathologies.

Up to date, the mechanisms by which *H. suis* might induce changes in VIP expression remain to be elucidated. However since alterations in VIP and its receptors have been reported in combination with gastric pathology (Erin et al., 2012), *H. suis*-induced gastric inflammation might be a possible explanation for the observed alterations in *VIP* and *VIPR1* expression upon *H. suis* infection.

VIP does not only contribute to maintenance of the gastrointestinal epithelial barrier integrity (Conlin et al., 2009), but it might also be involved in brain homeostasis. Indeed, fibers containing VIP have been identified close to the choroid plexus and it has been described that VIP regulates CSF secretion of the choroid plexus epithelial cells (Delgado et al., 2003; Nilsson et al., 1991; Lindvall et al., 1978). In addition, the protective role of VIP in neurodegenerative disorders, such as PD, has been reported (Delgado et al., 2003). Furthermore, it has been described that VIP is able to prevent overactivation of microglia in LPS-induced inflammation in the brain (Delgado et al., 2003; Kim et al., 2000). In our study, administration of VIP reduced LPS-induced blood-CSF barrier leakage and LPS-induced upregulation of *IL-6*, *TNF*, *KC* and *iNos* in the choroid plexus. Since VIP is present and produced in the CNS and the gastrointestinal system, its potential protective role in the

preservation of integrity of the epithelial barriers of gut and brain should not be neglected. The use of a VIP-knock-out mouse model might provide information on the role of VIP in *H. suis*-induced disruption of the integrity of the gut and brain barriers.

Taken together, our results suggest that increased gastrointestinal leakage and disturbance of the blood-CSF barrier along with neuroinflammation may be induced by *H. suis*-associated inflammation and dysregulation of the VIP signalling. Furthermore, the pivotal role of the choroid plexus in the communication between the stomach and the brain via systemic inflammation and dysregulation of VIP signalling has been identified. Nevertheless, further research is required to elucidate the underlying mechanisms by which an infection with *H. suis* may influence gastrointestinal–brain communication.

Optimization of diagnostic tools for the detection of *H. suis* in human samples

In general, diagnosing gastric NHPH infections in humans is problematic. Non-invasive commercial tests are currently unavailable. The urea breath test, frequently used to diagnose *H. pylori* infections, is usually negative in patients infected with NHPH, except when *H. pylori* co-exists (Matsui et al., 2014). The patchiness and sparseness of the gastric colonization by NHPH is probably the underlying cause for these false-negative results (Stolte et al., 1997; Solnick and Schauer, 2001). Serologic tests to screen for NHPH infections are lacking. Up until the present day, infection with these fastidious pathogenic agents is diagnosed by the analysis of gastric biopsies using histological, molecular and microbiological techniques (Debongnie et al., 1995; De Groote et al., 2001; Baele et al., 2004; O'Rourke et al., 2004; Matsui et al., 2014).

PCR has been considered as an analytically sensitive and specific diagnostic method for the identification of *Helicobacter* in human gastric biopsies (Ndip et al., 2003; Ricci et al., 2007). Moreover, real-time (RT)-PCR allows quantitation of bacteria in gastric biopsies (He et al., 2002). To identify *H. suis* in the gastric biopsy samples from the study performed in **chapter 1,** a *H. suis*-specific *ureA*-based SYBR green RT-PCR was used. For this latter molecular assay, additional sequencing of the positive amplicons was necessary, since cross-reactivity with host DNA and DNA from other gastric helicobacters occurred, questioning the biological specificity of this test. In SYBR green RT-PCR assays, a signal will be emitted when the fluorophore binds to any double stranded DNA (Valones et al., 2008). In our assay, aspecific binding of the *ureA*-based primers with other DNA than the *H. suis* target DNA will thus result in a fluorescent signal and in false-positive results. Furthermore, UreA is essential for all gastric helicobacters to resist the acidic environment and to colonize the stomach. This virulence factor is, based on its high sequence level identity among the different gastric helicobacters, well conserved (Smet et al., unpublished results) explaining the occurrence of cross-reactivity with DNA from closely related members in our assay. It can thus be concluded that the *ureA* gene is actually not the optimal candidate target for molecular assays in gastric *Helicobacter* diagnostics if identification to species level is required.

Recently, a new PCR has been developed, using primer pairs targeting the *H. suis carR* gene (Matsui et al., 2014). Based on BLASTN searches in the NCBI database, this gene is absent in other gastric and enterohepatic *Helicobacter* spp., *Campylobacter* spp. and several other bacteria including members of the *Enterobacteriaceae* family and might thus be, compared to *ureA*, a better candidate PCR amplicon for the identification of *H. suis*

by reducing the possibility for cross-reactivity with DNA from other bacteria (Matsui et al., 2014). Sensitivity and specificity of this *carR*-based molecular assay was at the onset of this thesis lacking and determination of both parameters is essential for its introduction into routine clinical use. Therefore, in the **third chapter** of this thesis, we developed a *carR* probe-based RT-PCR that allows detection and quantification of *H. suis* in gastric tissue samples. The introduction of a *H. suis*-specific *carR* probe increased the specificity by excluding cross-reactivity with host DNA and DNA from other bacteria. Such probe will bind to a specific region within the single-stranded DNA. In contrast to SYBR green assays, in probe-based assays fluorescence will only be emitted when the DNA polymerase separates the reported dye from the quencher during amplification, rendering the probe-based RT-PCRs more specific (Valones et al., 2008). Our assay validation showed a high degree of diagnostic specificity and sensitivity compared to the *ureA*-based SYBR green RT-PCR. However, further application on large numbers of clinical samples is recommended. The optimization of such assay is a major progress in *H. suis* diagnostics in humans and similar assays should be developed for the other zoonotic NHPH species as well. Screening and comparison of the recently available NHPH genome sequences (Vermoote et al., 2011; Schott et al., 2011; Arnold et al., 2011; Smet et al., 2013; Smet et al., unpublished results) might facilitate the search for and identification of potential *Helicobacter* species-specific genes. Subsequently, PCR-based molecular diagnostic tools based on the amplification of specific regions within these potential *Helicobacter* species-specific genes may be developed.

Another method to identify gastric *Helicobacter* infections in gastric biopsies is isolation and cultivation of *Helicobacter.* For *H. pylori,* the success rate for cultivation reaches up to 100% (Ndip et al., 2003), but it depends on the quality, the quantity and the transportation conditions of the gastric biopsies (Ricci et al., 2007). Because of the high specificity and sensitivity, cultivation is considered as the gold standard for the diagnosis of *H. pylori* infections (Kusters et al., 2006). For NHPH, however, this method has not yet been successful, with *H. felis* and *H. bizzozzeronii* being the only 2 species cultivated so far from the human stomach (Kivisto et al., 2010; Wüppenhorst et al., 2013). This can mainly be attributed to their very fastidious nature (Haesebrouck et al., 2009). For *H. suis*, it requires a highly enriched biphasic medium at pH 5 and a microaerobic atmosphere (Baele et al., 2008). Isolation of this agent from the gastric mucosa of pigs is possible but is a long and laborious process due to its slow growth. The risk of overgrowth with other bacteria makes frequent subculturing necessary in order to obtain pure cultures and it usually takes several weeks to obtain a single *H. suis* isolate (Baele et al., 2008). Efforts to isolate *H. suis* from human gastric biopsies, either directly (unpublished results) or after mouse-passage (Matsui et al., 2104), remained unsuccessful. The low colonization density of this pathogenic agent in the human stomach (Matsui et al., 2014) as well as the relative fast overgrowth of the isolation plates with other members of the gastric microbiome, including *Lactobacillus* spp., have no doubt contributed to this failure (Sgouras et al., 2004; Vilaichone et al., 2002; Chenoll et al., 2011; Matsui et al., 2014). Furthermore, previous studies have described the inhibitory effects on the growth and colonization capacity of *H. pylori* by lactobacilli and bifidobacteria in the stomach (Vilaichone et al., 2002; Sgouras et al., 2004; Francavilla et al., 2008; Medouakh et al., 2010; Aiba et al., 2015; Chenoll et al., 2011). This may also be the case for *H. suis in vitro.* To overcome these obstacles, we improved the mouse-passage-based protocol for the isolation of low numbers of *H. suis* bacteria. Mouse-passage of *H. suis*-infected gastric biopsies

might allow *H. suis* to multiply and to increase in numbers which might increase the chances for successful isolation. In our study, *H. suis* was successfully re-isolated from the stomach of mice on a biphasic medium, specified for this bacterium as described before (Baele et al., 2008), and additionally supplemented with 5 µg/ml linezolid. This antimicrobial agent is active against a broad spectrum of Gram-positive bacteria, including many *Lactobacillus* species (Yagi and Zurenko, 1997; Agar and Gold, 2012), and has a relatively low activity (MIC_{90} 8µg/ml) against *Helicobacter* (Sanchez et al., 2000). Indeed, in the presence of linezolid, *H. suis* was able to grow whereas lactobacilli proliferation was clearly inhibited. This optimized isolation protocol can now be applied to isolate *H. suis* from human gastric biopsies. A possible drawback is that mice should be inoculated with homogenized gastric tissue containing 10^4 bacteria/ml. Indeed, in our study, the minimal infection dose of *H. suis* in mice was 10^4 viable *H. suis* bacteria/ml. The same infection dose has been described before and has also been reported for *H. pylori* infection in rhesus monkeys (Matsui et al., 2014; Solnick et al., 2001). Nevertheless, due to the low colonization density and patchy distribution of *H. suis* in the human stomach, this bacterium can still be missed when collecting biopsies. Pooling gastric biopsies obtained from several regions in the stomach or taking gastric brushes might counter this problem.

Isolation of human-derived *H. suis* would be a major breakthrough in *H. suis*-related research. First, the availability of such isolates would allow *in vitro* antimicrobial susceptibility testing. Because the increasing development of antibiotic resistance in *Helicobacter* species has been suggested (Van de Bulck et al., 2005; Kusters et al., 2001; Megraud et al., 2004; Megraud et al., 2007; Vermoote et al., 2011; Thung et al., 2016; Regnath et al., 2016), antimicrobial susceptibility testing of human *H. suis* isolates would allow to determine an appropriate patient-specific eradication therapy, thereby minimizing the occurrence of treatment failures and antibiotic resistance (Thung et al., 2016). This has already been applied to eradicate *H. pylori* infections in humans (Draeger et al., 2015). DNA prepared from pure human-derived *H. suis* isolates could also be used to further characterize this agent and to identify the origin and the evolution of these strains by comparing their genomes with those from porcine and non-human primate *H. suis* strains (Vermoote et al., 2011; Liang et al., 2015; Joosten et al., 2013; Bosschem et al., 2016; Flahou et al., unpublished data). Finally, to study the pathogenesis of gastric *H. suis* infections, experimental infection studies are indispensable. Such *in vivo* trials have already been performed in rodent models using porcine and non-human primate strains (Flahou et al., 2010; Bosschem et al., 2016). Also infection studies with human-derived *H. suis* have been carried out in the past (O'Rourke et al., 2004; Nakamura et al., 2007; Park et al., 2008; Matsui et al., 2014). However, in those mouse experiments, homogenized human gastric tissue was used as inoculum. This implies that other microorganisms were inoculated together with *H. suis*, which might have influenced the results (Flahou et al., 2010). Infection of laboratory animals with pure human-derived *H. suis* isolates might therefore provide a more accurate image of the pathogenesis of infections with this fastidious bacterium (O'Rourke et al., 2004; Nakamura et al., 2007; Flahou et al., 2010; Matsui et al., 2014).

Taken together, the developed methods may be implemented for diagnosis of *H. suis* in human-derived gastric biopsies. The probe-based RT-PCR can be useful for the detection of *H. suis* bacteria in the human stomach, whereas the application of the optimized mouse-passage-based isolation protocol might enhance the chance to obtain and characterize human *H. suis* isolates and to study their pathogenesis *in vivo*.

Final conclusions and future perspectives

In conclusion, we observed an increased frequency of gastric *H. suis* infections in human patients with PD, suggesting a possible link between *H. suis* infection and PD. This was further substantiated by demonstrating that an infection with this microorganism may influence the stomach-brain axis via the systemic circulation and may affect VIP signaling. Furthermore, the availability of optimized and accurate diagnostic tools to identify *H. suis* infections opens new perspectives for the study of its true prevalence in the human population.

It should be further investigated whether the observed effects of a gastric *H. suis* infection on the brain barriers will sensitize to or enhance the development of PD. This can be determined by the use of different *in vivo* models. PD can be mimicked in mice by intracranial injection of the neurotoxin 6-hydroxydopamine or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridin (MPTP) or by using A30P α-synuclein transgenic mouse models, overexpressing the human A30P α-synuclein gene (Godoy et al., 2008; Plaas et al., 2008; Antony et al., 2011). However, both 6-hydroxydopamine and MPTP have been reported to cause impairment of the BBB (Carvey et al., 2005; Chen et al., 2008), suggesting that the use of the A30P α-synuclein transgenic mouse models is more reliable for the evaluation of the effect of *H. suis* on the course of PD. On the other hand, Sampson et al. (2016) recently described the importance of the gut microbiota for the induction of motor deficits, microglia activation and α-synuclein pathology in α-synuclein overexpressing transgenic mouse. Indeed, antibiotic treatment improved pathophysiology in adult animals, whereas the pathophysiology was increased by microbial re-colonization of the animals.

It might also be interesting to perform similar experimental trials to evaluate the influence of *H. suis* infections on development and/or progression of other neurodegenerative disorders. For example, the influence of *H. suis* on Alzheimer's disease, the most common neurodegenerative disorder in the current human population (Wirdefelt et al., 2011), may be studied by using rodent models. Alzheimer's disease can be mimicked by intrahippocampal injection with the neurotoxin amyloid β, which induces the formation of Aβ-plaques in the brain, or by using APP-PS1 transgenic mouse models, overexpressing human amyloid β (Lok et al., 2013; Jean et al., 2015).

Besides studying the integrity of the stomach-brain barriers, loss of dopaminergic neurons in the substantia nigra of the brain should be investigated. This can be done by using tyrosine hydroxylase staining with or without a silver nucleolar staining technique (Healy-stoffel et al., 2012). Additionally, the presence of lewy bodies in the remaining neurons can be determined by immunohistochemical stainings such as the α-synuclein immunohistochemical staining (Stefanis, 2012) or the ubiquitin immunohistochemical staining (Chu et al., 2000). Also, expression of genes associated with PD, such as *α-synuclein, parkin* (*PARK*)*, phosphatase and tensin homolog* (*PTEN*)*-induced putative kinase 1* (*PINK*) and *mitochondrial ribosomal protein S6* (*MRPS6*) genes, can be measured in the brain (Lewis et al., 2012).

Apart from impairment of the stomach-brain axis resulting in systemic inflammation and dysregulation of the VIP expression, changes in the intestinal microbiome might also play a role in development of PD and/or influence its course. Several studies have indeed described a possible link between such changes and brain

disorders, including depression, autism, Alzheimer's disease and PD (Parracho et al., 2005; Bercik et al., 2011; Bravo et al., 2011; Zhang et al., 2015; Hu et al., 2016; Mulak et al., 2015). Changes in the gut microbiome may be caused by a broad range of triggers, including gastrointestinal infections (Ochoa-Reparaz and Kasper, 2014; Walters et al., 2014; Zhang et al., 2015). *H. pylori* infections have already been shown to affect the gastrointestinal microbiota in humans (Engstrand et al., 2013; Budzynski et al., 2014; He et al., 2016). Therefore, it might be recommended to evaluate the effect of an *H. suis* infection on the gut microbiome, for instance in experimentally infected mice and in *H. suis* infected human patients, before and after eradication of the infection.

Additionally, it might be recommended to evaluate wether the induced changes in brain homeostasis are directly due to the actual infection with *H. suis,* or rather to possible *H. suis*-induced changes in the gut microbiome. Intestinal microbiota transplantation from a *H. suis* infected mice to the intestinal tract of a control recipient in order to change the recipient's gut microbial composition might be useful here (Gupta et al., 2016). If it appears that the shift in the composition of the microbiota is associated with changes in brain homeostasis, intestinal microbiota transplantation from a healty person to a patient suffering fom a neurodegenerative disorder might be a potential therapeutic strategy. Indeed, intestinal microbial transplantation has already been suggested as a possible therapy for treatment of IBD, diarrhea, *Clostridium difficile* infections, etc. (Gupta et al., 2016).

Our results indicate that the administration of VIP might play a protective role in inflammation-induced disruption of the blood-CSF barrier integrity and brain homeostasis. The immunomodulatory and neuroprotective effect of VIP has already been suggested in the past (Kim et al., 2000; Delgado and Ganea, 2003; Delgado et al., 2004). Moreover, a protective effect of VIP in PD and Alzheimer's disease has been described (Delgatdo and ganea, 2003; Song et al., 2012). Therefore, the administration of VIP might be another possible therapeutic strategy in patients suffering from PD or other neurodegenerative disorders.

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SUMMARY

Helicobacter pylori is the most prevalent *Helicobacter* species in humans. Infection with this agent has been associated with a broad range of gastric disorders, including gastritis, ulcers and cancer. Besides causing inflammation in the stomach, *H. pylori* infection has also been linked to extragastric manifestations, including Parkinson's Disease (PD). Patients with this neurodegenerative disorder frequently display gastrointestinal complaints but their symptoms cannot always be assigned to infection with *H. pylori*.

Gastric disease in humans has, however, also been associated with spiral-shaped non-*Helicobacter pylori Helicobacter* (NHPH) species, such as *H. suis.* This zoonotic agent, highly prevalent in pigs and non-human primates, is the most prevalent NHPH in humans and has been associated with gastritis, gastric and duodenal ulcers and mucosa-associated lymphoid tissue (MALT) lymphoma. The risk for developing MALT lymphoma is higher when infected with *H. suis* than *H. pylori*. Living in close contact with pigs or the consumption of raw or undercooked meat are considered to be risk factors for infection with this agent.

Compared to *H. pylori*, diagnosis of *H. suis* infections is extremely difficult due to the sparse and patchy distribution in the human stomach and the fastidious nature of this bacterium.

At the onset of this thesis, no information was available on the prevalence of *H. suis* in Parkinson's disease patients, nor was there any information found on how and to what extend an infection with this agent could contribute to the outcome of this neurodegenerative disorder. Additionally, the currently used methods to diagnose *H. suis* infections, lacked optimization in terms of sensitivity and specificity.

In our first study, we investigated the prevalence of *H. suis* infections in patients suffering from idiopatic parkinsonism (IP) and gastric complaints and compared it with the known frequency of *H. pylori* infection in this group. A control group of gastroenterology patients with no clinical parkinsonism was included for the purpose of comparison. Archived DNA extracts were examined for the presence of *H. suis* DNA using an *ureA*-based SYBR green RT-PCR assay. Results were confirmed by additional sequencing of the positive PCR amplicons. Additionally, multi-locus sequence typing (MLST) was performed to demonstrate that the *H. suis* DNA in the biopsy extracts was different from that of the *H. suis* lab strain used as positive control in the RT-PCR assay.

From the 60 patients with IP, 27% and 28% tested positive for *H. suis* and *H. pylori* respectively, whereas only 2% and 16% from the 256 control patients tested positive for *H. suis* and *H. pylori,* respectively. These results indicated that the relative risk of having an infection with *H. suis* was 10 times higher in PD patients compared to controls. Interestingly, from the 16 *H. suis* positive IP patients, 11 were from 19 with proven *H. pylori* eradication, 3 from 17 pre-*H. pylori* eradication and 2 from 24 *H. pylori* culture/PCR negative. Remarkably, the frequency of *H. suis* was the highest in the patients where *H. pylori* has been eradicated.

Partial MLST results for the *H. suis* DNA found in extracts from 6 IP patients and 1 control showed that the allele pattern in these samples differed from that of the DNA from the *in vitro* lab strain. This indicated that the results were not due to contamination.

In conclusion, this study highlighted that the prevalence of *H. suis* was higher in patients with IP compared to our control group and that it was even more exaggerated in those following *H. pylori* eradication therapy.

The brain is protected from alterations in systemic circulation by a series of specialized barriers, including the blood-brain barrier (BBB) and the blood cerebrospinal fluid barrier (blood-CSF barrier). Dysfunction of these barriers has been linked to multiple neurodegenerative disorders, including Parkinson's disease. Furthermore, systemic inflammation might contribute to the impairment of the BBB and the blood-CSF barrier. Since infections with *Helicobacter* have been suggested to induce systemic inflammation, it might be possible that a gastric *H. suis* infection could influence the integrity of the brain barriers and thereby contribute in the pathophysiology of neurodegenerative disorders. Therefore, **in the second study**, we evaluated the influence of an experimental infection with *H. suis* on the integrity of the stomach-brain axis in mice. To investigated this, C57BL/6 mice were inoculated intragastrically with viable *H. suis* and compared to a negative control group. At 4 weeks post-infection, animals were euthanized and samples from the stomach and brain were analyzed. An infection with *H. suis* induced gastric inflammation, characterized by the infiltration of mononuclear and polymorphonuclear cells in the mucosa and submucosa of the stomach. This finding was accompanied by the increased expression of interleukin *(IL)-1β*, murine *IL-8* homologs and increased protein levels of IL-1β and IL-17. A significant increase in the gastric mucosal permeability was associated with abberrant expression of the tight junction protein zonula occludens *(ZO)-1* and the transmembrane *Muc13* mucin. Furthermore, altered expression of the vasoactive intestinal peptide (*VIP*) and its receptor (*VIPR1*) was detected in the stomach of *H. suis*-infected mice. VIP has been shown to play a regulatory and protective role in barrier integrity, tight junction expression and mucin secretion in the gastrointestinal tract. These findings indicated that the loss of gastric-mucosal barrier integrity in *H. suis* infected mice might result in increased gastrointestinal leakage of cytokines, chemokines and bacterial compounds which could lead to systemic inflammation. Indeed, increased levels of IL-1β and the murine IL-8 homologue, MIP1α were observed in the serum of infected animals.

Subsequently, the influence of *H. suis* on the integrity of the brain barriers (BBB and blood-CSF barrier) and brain homeostasis was investigated. A significant increase in the blood-cerebrospinal fluid (CSF) barrier permeability upon *H. suis* infection was seen. In contrast, blood-brain barrier permeability was unaffected at this acute stage of infection. Disturbance of the blood-CSF barrier was associated with an increased expression of the pro-inflammatory cytokine *Il-1*β and the inducible nitric oxide synthase (*iNos*) and a decreased expression of the tight junction genes of claudin 1 (*clnd-1*) and *Occludin* (*Ocln*) in the choroid plexus epithelium that forms the blood-CSF barrier. Additionally, an increase of *IL-6* expression in the hippocampus was observed upon infection with *H. suis.* These changes in cytokine expression in the brain were accompanied by the activation of microglia cells in *H. suis*-infected animals. Apart from the involvement of VIP in the maintenance of the gastrointestinal epithelial barrier, this neuropeptide also plays a role in brain homeostasis. Systemic administration of VIP indeed reversed the inflammation-induced disruption of the blood-CSF barrier integrity.

In conclusion, we have identified the pivotal role of the choroid plexus in the communication between the gastrointestinal tract and the brain. Furthermore, our results indicate that disruption of the blood-CSF barrier is induced by *H. suis*-associated systemic inflammation and/or dysregulated VIP signaling.

Diagnosing gastric NHPH infections, in general, in humans is problematic. Non-invasive commercial tests are currently unavailable. Nowadays, diagnosis of infection with these fastidious pathogenic agents has been performed by the analysis of gastric biopsies using histological, molecular and microbiological techniques. To identify *H. suis* in the gastric biopsy samples, a *H. suis*-specific *ureA*-based SYBR green RT-PCR has been frequently used. For this molecular assay, additional sequencing of the positive amplicons is necessary, since cross-reactivity with host DNA and DNA from other gastric helicobacters occurred, questioning the specificity of this test. Attempts to directly and/or indirectly (after mouse-passage) isolate pure *H. suis* strains from human gastric biopsies remained unsuccessful. Direct isolation is hampered by the fastidious nature, the low colonization density and the patchy distribution of this pathogen, whereas indirect isolation is hampered by overgrowth with gastric contaminants, including *Lactobacillus* spp. In the **third study** of this thesis, we first developed a probe-based RT-PCR for the detection and quantitation of *H. suis* DNA in gastric biopsies. This RT-PCR was based on the amplification of the *H. suis*-specific *carR* gene. The probe detects a well-conserved 20bp long region within this gene. Validation of the assay revealed a high degree of specificity and sensitivity, high linear correlations (r^2 between 0.995-0.999) and high amplification efficiencies (>90%). In order to exclude cross-reaction with host DNA, gastric DNA extracts from human patients, pigs, mice and non-human primates were analyzed. In contrast to the *ureA*-based RT-PCR, no aspecific interactions with human, porcine, murine and non-human primate DNA, nor with DNA from other bacteria, including *Helicobacter s*pp. and *Campylobacter* spp., were detected when using the *carR*-probe based RT-PCR.

Secondly, a mouse-passage-based protocol was further optimized for the isolation of low numbers of viable *H. suis* bacteria. Mice were inoculated with different numbers of viable *H. suis* bacteria (10^2-10^8). The animals were kept for 4 weeks to allow colonization and multiplication of *H. suis.* In order to inhibit the proliferation of gastric contaminants, including *Lactobacillus* spp., from the murine stomach, 5µg/ml linezolid was added to a biphasic medium consisting of *Brucella* agar with *Brucella* broth on top of it, both supplemented with vitox supplement, *Campylobacter*-selective supplement, amphotericin (5µg/ml), HCl (0.05%) and fetal bovine serum (20%). Using the optimized isolation protocol, we were able to re-isolate *H. suis* from the stomach of mice infected with at least 10^4 viable *H. suis* bacteria.

Finally, we also sequenced the genome of the porcine *H. suis* strain before and after mouse-passage in order to determine the influence of mouse-passage on the genome of *H. suis.* Our data shows that mouse-passage of *H. suis* does not result in major recombination events within the genome of this bacterium, suggesting that *H. suis s*trains obtained after mouse-passage can be used for further characterization of this pathogen.

In conclusion, the methods described above can be implemented for detection or isolation of *H. suis* from human gastric biopsies.

Samenvatting

Helicobacter pylori is de meest voorkomende *Helicobacter* soort bij de mens. Deze kiem wordt geassocieerd met gastritis, peptische ulcera en maagkanker. Daarnaast worden infecties met *H. pylori* ook gelinkt aan andere aandoeningen, zoals de ziekte van Parkinson. Patiënten met de ziekte van Parkinson vertonen vaak maagklachten, maar hun symptomen kunnen niet altijd toegeschreven worden aan een infectie met deze pathogeen. Maagaandoeningen bij de mens kunnen ook veroorzaakt worden door infecties met niet-*H. pylori Helicobacter* species, zoals *H. suis* die van nature uit de maag van varkens en primaten koloniseert. Bij de mens veroorzaakt dit zoönotisch agens gastritis, peptische ulcera en lymfomen van het lymfoïd weefsel van de maagmucosa (MALT-lymfomen). Bovendien is het risico op de ontwikkeling van MALT-lymfomen groter bij een *H. suis* infectie dan bij een infectie met *H. pylori*. Mensen die in nauw contact leven met varkens of rauw/niet doorbakken varkensvlees consumeren, hebben meer kans om besmet te worden met *H. suis*.
De diagnose van infecties met *H. suis* bij de mens is moeilijker in vergelijking met *H. pylori* omwille van het zeer focale kolonisatiepatroon in de maag en de hoge moeilijkheidsgraad om dit agens *in vitro* te cultiveren. Bij de start van dit doctoraatsonderzoek was er nog geen informatie beschikbaar over het voorkomen van *H. suis* infecties bij patiënten met de ziekte van Parkinson. Het was eveneens nog onduidelijk of en in welke mate een infectie met deze pathogeen kan bijdragen tot het ziektebeeld van deze neurodegeneratieve aandoening. Bovendien bleek dat de aangewende technieken voor de detectie van *H. suis* infecties bij de mens niet optimaal waren.

In een **eerste studie** werd het voorkomen van *H. suis* infecties bestudeerd bij patiënten met de ziekte van Parkinson die maagklachten hadden en vervolgens vergeleken met de reeds gekende prevalentie van *H. pylori* binnen deze groep. Een controle groep (patiënten met maagklachten, maar zonder de ziekte van Parkinson) werd eveneens ingesloten ter vergelijking. Het DNA van de maagstalen werd onderzocht op de aanwezigheid van *H. suis* DNA aan de hand van een op het *ureA*-gen gebaseerde RT-PCR analyse en sequenering van de positieve PCR amplicons. Multi-locus sequence typing (MLST) werd uitgevoerd om na te gaan of het *H. suis* DNA in de maagbiopten verschillend was van dat van de *H. suis* laboratorium stam die gebruikt werd als positieve controle.
In de groep van patiënten met de ziekte van Parkinson testte 27% positief voor *H. suis* en 28% voor *H. pylori*. In de controle groep werd *H. suis* slechts aangetoond in 2% van de patiënten, terwijl 16% van de patiënten geïnfecteerd waren met *H. pylori*. Deze resultaten tonen aan dat een *H. suis* infectie 10 maal frequenter voorkwam bij patiënten met de ziekte van Parkinson in vergelijking met de controle groep. Bovendien bleek dat de meeste patiënten met de ziekte van Parkinson die positief testten voor *H. suis,* een succesvolle *H. pylori* eradicatie therapie achter de rug hadden. Tot slot bleek uit de MLST resultaten dat het *H. suis* DNA in de maagbiopten verschillend was van dat van de laboratorium stam waardoor contaminatie kon uitgesloten worden.
Samengevat beschreven we in deze studie een opmerkelijk hoge prevalentie van *H. suis* infecties bij patiënten met de ziekte van Parkinson en voornamelijk bij de patiënten die voorafgaand een anti-*H. pylori* therapie hadden ondergaan.

De hersenen worden beschermd tegen gevaarlijke stoffen in de bloedcirculatie door een aantal gespecialiseerde barrières, zoals de bloed-hersen barrière (BBB) en de bloed-cerebrospinaal vocht barrière (bloed-CSF barrière). Bij verstoring van deze barrières kunnen cytokines en chemokines vanuit de bloedbaan de hersenen binnendringen en inflammatie induceren. Er werd inderdaad reeds aangetoond dat systemische inflammatie een belangrijke rol speelt in de ontwikkeling van de ziekte van Parkinson. Bovendien doodt *H. suis* de epitheliale cellen in de maag en wordt de epitheelbarrière ter hoogte van de maag doorbroken, waardoor ontstekingsmediatoren ook in de circulatie kunnen terechtkomen. Daarom werd er tijdens **de tweede studie** van deze thesis nagegaan in welke mate en op welke manier een *H. suis* infectie de BBB en de bloed-CSF barrière beïnvloedt. Hiervoor werden C57BL/6 wildtype muizen intragastraal geïnoculeerd met levende *H. suis* bacteriën of met het opgroeimedium van deze kiem (negatieve controle). Vier weken na infectie werden de dieren geëuthanaseerd en stalen van de maag en de hersenen werden verzameld en geanalyseerd. Inflammatie ter hoogte van de maag werd gekenmerkt door de infiltratie van mononucleaire en polymorfonucleaire cellen in de mucosa en de submucosa van de maag en een verhoogde mRNA en/of eiwit expressie van interleukine (IL)-1β, IL-17 en de chemokines KC en LIX, die bij muizen tot expressie worden gebracht en homoloog zijn aan IL-8 van mensen. Een verhoogde maagpermeabiliteit werd geassocieerd met veranderingen in mRNA en eiwit expressie van het transmembranair Muc13 mucine en het tight junction eiwit zonulae occludens (ZO)-1. Bij de met *H. suis* geïnfecteerde dieren werd tevens een onderdrukking van de expressie van het vasoactief intestinaal peptide (VIP) vastgesteld. Dit peptide speelt een belangrijke rol in het behoud van de maagmucosa integriteit. Een verstoorde mucosale maagbarrière kan leiden tot lekkage van cytokines, chemokines en bacteriële componenten vanuit de maag naar de bloedsomloop en kan dus resulteren in systemische inflammatie. In deze studie werden in het serum van de met *H. suis* geïnfecteerde muizen verhoogde titers gedetecteerd van IL-1β en MIP1α, een cytokine dat net als KC en LIX door muizen tot expressie wordt gebracht en homoloog is aan IL-8 van mensen. Vervolgens werd de impact van een *H. suis* infectie op de integriteit van de hersenbarrières onderzocht. Er werd een significante stijging in de bloed-CSF barrière permeabiliteit waargenomen. De permeabiliteit van de BBB, daarentegen, werd niet aangetast in dit acuut stadium van de infectie. De verstoring van de bloed-CSF barrière werd geassocieerd met een verhoogde expressie van *IL-1β* en het induceerbare stikstof oxide synthase (*iNos*), alsook met een gedaalde expressie van de tight junctions claudine 1 (*clnd-1*) en occludine (*Ocln*) ter hoogte van het choroid plexus epitheel dat de bloed-CFS barrière vormt. Tevens werd in de hippocampus van de met *H. suis* geïnfecteerde dieren een verhoogde expressie waargenomen van het gen dat codeert voor IL-6. De veranderingen in de expressie van cytokines in de hersenen gingen eveneens gepaard met een activatie van de microgliacellen bij *H. suis* geïnfecteerde muizen.

VIP zou niet enkel een rol spelen in het behoud van de integriteit van de maagmucosa, maar eveneens in het behoud van de homeostase in de hersenen. Vermits de expressie van VIP verstoord was in de maag van met *H. suis* geïnfecteerde dieren, wilden we in deze studie het effect van VIP op de hersenen nagaan. Hiervoor werden muizen intraperitoneaal geïnjecteerd met lipopolysaccharide, wat een ontstekingsreactie en een verstoring van bloed-CSF barrière veroorzaakt. Systemische toediening van VIP resulteerde in een herstel van de verstoorde bloed-CSF barrière.

Samengevat, werd in deze studie aangetoond dat de choroid plexus een centrale rol speelt in de communicatie tussen de maag en de hersenen . Bovendien wijzen onze resultaten erop dat een verstoorde bloed-CSF barrière het gevolg kan zijn van een door *H. suis*-geïnduceerde systemische inflammatie en/of een verstoring in de VIP huishuiding.

De diagnose van infecties met niet-*H. pylori Helicobacter* species is problematisch en tot op heden zijn er nog geen commerciële testen beschikbaar. Voor het aantonen van *H. suis* DNA in stalen van de maag wordt er o.a. gebruik gemaakt van een SYBR green RT-PCR techniek gebaseerd op het *ureA* gen van dit agens. Bijkomende sequenering van de positieve PCR amplicons is echter noodzakelijk omwille van het voorkomen van kruisreactiviteit met humaan DNA en DNA van andere gastrale *Helicobacter* species. Daarnaast is men er nog niet in geslaagd om *H. suis* te isoleren uit maagbiopten (rechtstreeks of via passage in een muismodel) en te cultiveren *in vitro*. In de **derde studie** werd dan ook eerst een nieuwe RT-PCR ontwikkeld voor de detectie en kwantificatie van *H. suis* DNA in maagbiopten. De RT-PCR was gebaseerd op de amplificatie van het *H. suis*-specifieke *carR* gen. Om amplicons te detecteren werd er, in plaats van het frequent gebruikte SYBR green, geopteerd voor een fluorescent gemerkte probe die een geconserveerde regio van 20 baseparen binnen het *carR* gen detecteert. Validatie van deze techniek toonde een hoge specificiteit en sensitiviteit aan, alsook hoge lineaire correlaties (r^2 tussen 0.995-0.999) en een hoge amplificatie efficiëntie (>90%). Er werd geen kruisreactie vastgesteld met DNA van andere bacteriën, humane patiënten, varkens, muizen en niet-humane primaten.

Vervolgens werd een bestaand protocol voor de isolatie van lage aantallen levende *H. suis* bacteriën uit de maag via passage in een muismodel verder geoptimaliseerd. Hiervoor werden muizen intragastraal geïnoculeerd met verschillende concentraties levende *H. suis* kiemen (10^8-10^2). De dieren werden gedurende 4 weken aangehouden om kolonisatie en vermeerdering van *H. suis* mogelijk te maken. Daarna werden ze geëuthanaseerd en de maag werd voorbehandeld met een 1% HCl oplossing gedurende 45 minuten. Deze voorbehandelde stalen werden dan geïnoculeerd in het bifasische opgroeimedium van *H. suis* waaraan 5µg/ml linezolid was toegevoegd. Dit antibioticum is nodig om te vermijden dat bacteriën die behoren tot de microbiota van de muizenmaag, waaronder *Lactobacillus* spp., het medium overwoekeren en isolatie van *H. suis* onmogelijk maken. Het opgroeimedium bestond uit *Brucella* agar met daarboven *Brucella* broth, beiden gesupplementeerd met Vitox, *Campylobacter*-selectief supplement, amphotericine (5µg/ml), HCl (0,05%) en geïnactiveerd foetaal kalfserum (20%). Door gebruik te maken van het geoptimaliseerde isolatie protocol slaagden we erin om *H. suis* te isoleren uit de maag van muizen die initieel werden geïnfecteerd met tenminste 10^4 levende *H. suis* kiemen.

Tot slot werd er ook nagegaan of de passage van *H. suis* in de muizenmaag een impact heeft op het genoom van deze bacterie. Hiervoor werd het genoom van de *H. suis* stam voor en na isolatie uit de muizenmaag gesequeneerd. Uit de genoomanalyses konden geen grote veranderingen in het genoom waargenomen worden. Samengevat kunnen we stellen dat de ontwikkelde en geoptimaliseerde diagnostische technieken gebruikt kunnen worden voor de detectie en/of isolatie van *H. suis* in humane maagbiopten.

CURRICULUM VITAE

Caroline Blaecher werd geboren op 18 januari 1988 te Brussel. Nadat ze haar secundair onderwijs aan het Jan van Ruusbroeckollege succesvol had afgerond, startte ze in 2006 de studies diergeneeskunde aan de Universiteit Gent. In 2012 behaalde ze met onderscheiding het diploma van Master in de Diergeneeskunde.

Omwille van haar grote interesse in het wetenschappelijk onderzoek, startte ze onmiddellijk daarna een doctoraatsonderzoek bij de Vakgroep Pathologie, Bacteriologie en Pluimveeziekte aan de Faculteit Diergeneeskunde van de Universiteit Gent. Gedurende 4 jaar verrichtte ze, in het kader van een Geconcerteerde Onderzoeksactie (GOA) van de Universiteit Gent (Code GOA 01G00408) en de "Psychiatry Research Trust" (Registered Charity no. 284286) van Londen, onderzoek naar de mogelijke associatie tussen *Helicobacter suis* infecties en de ziekte van Parkinson en optimaliseerde ze diagnostische testen voor de detectie van deze infecties bij de mens. Dit doctoraatsonderzoek resulteerde in het huidige proefschrift.

Caroline Blaecher is auteur en medeauteur van verschillende publicaties in internationale wetenschappelijke tijdschriften. Ze nam eveneens actief deel aan nationale en internationale congressen en was daar meermaals spreker.

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